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PRINCIPAL INVESTIGATOR:
Andrew J. Armstrong, MD ScM

CONTRACTING ORGANIZATION:
Duke University
Durham, NC 27705-4677

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Andrew J Armstrong, MD ScM

Email: andrew.armstrong@duke.edu

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14. ABSTRACT
The purpose of this DOD NIA is to develop a novel method to detect circulating tumor cells (CTCs) from men with metastatic castration resistant prostate cancer (CRPC) based on a range of CTC phenotypes. The novel method employs a nanoparticle polymersome that contains near-infrared emissive porphyrins and permits antibody conjugation for target engagement and flow sorting in the infrared spectrum for specificity. We are developing near infrared emissive polymersomes (NIR-EPs) that contain antibodies to EpCAM, N-cadherin, OB-cadherin, and PSMA which permit the isolation and enumeration ev vivo of CTCs bearing these antigens in the circulation of men with CRPC. These specialized CTC detection nanoparticles permit the isolation of specific CTC phenotypes including epithelial, mesenchymal, and prostate cancer specific targets. In year 2 we have continued to optimize the methodology and chemistry for the creation of these NIR-EPs, including chemical synthesis, antibody conjugation, optimal reagents and processing, positive and negative control cell applications, and methods for red cell lysis, leukocyte depletion, and flow sorting of CTCs in the infrared spectrum. Challenges have included specificity due to antibody affinity during conjugation. This work is ongoing in the Therien laboratory to provide a clinical reagent for the ex vivo capture and identification of CTCs without binding to leukocytes nonspecifically. A 6 month no cost extension was requested to continue this optimization process.

15. SUBJECT TERMS- Circulating tumor cells, prostate cancer, epithelial plasticity, epithelial mesenchymal transition, metastasis, lethal phenotype, polymersome, nanoparticle, phenotype

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INTRODUCTION

In 2013-14, over 80 U.S. men will die every day from metastatic prostate cancer (PC).[1] Many deaths could potentially be prevented or delayed through identification and treatment directed at high risk disease prior to the development of metastases. Currently, clinical/pathologic measures (i.e. PSA, stage, grade) provide little biologic insight into the process by which PC cells metastasize and become lethal. The measurement of circulating tumor cells (CTCs) in men with PC represents one biomarker with prognostic and predictive implications.[2] Many patients with metastatic PC, however, have undetectable CTCs, limiting clinical utility. We have identified epithelial-mesenchymal transitions (EMT) in experimental models of PC in which the cellular phenotype undergoes reversible (plastic) changes from an epithelial to a mesenchymal nature facilitating metastatic spread, followed by epithelial reversion in the target metastatic organ.[3] While in the active process of metastasis, CTCs may possess a mesenchymal/plastic phenotype, and thus may not be captured by existing epithelial-based CTC technologies.

We are developing a novel CTC capture method, termed the near-infrared emissive polymersome (NIR-EP) which permits antibody conjugation to this light-emissive nanoparticle for tumor-specific binding and sorting from normal blood cells. In this DOD IDA/NIA 2014 annual report, we provide an update on our progress to develop NIR-EPs capable of binding prostate cancer cells with a range of phenotypes, to distinguish these cells from normal leukocytes, to isolate these cells using flow sorting based on near-infrared emission spectra, and to customize these nanoparticles based on the target cancer protein of interest. In year 2 we have reevaluated our protocols for the fabrication of antibody-conjugated NIR-EPS, to optimize performance characteristics for NIR-EPS against EpCAM, N-cadherin, O-cadherin and PSMA for the isolation of CTCs, and particularly for cells that have lost EpCAM expression. These efforts have delayed testing of these NIR-EPs in healthy volunteers and men with metastatic castration resistant prostate cancer to provide proof of principle that these NIR-EPs provide similar or greater isolation of CTCs as compared with conventional ferrofluid-based assays such as the Veridex Cellsearch test. However, we are now confident that with our optimized protocols, we will be able to rapidly and reproducibly be able to generate these materials to provide insight into metastasis biology in PC and lead to the identification of relevant targets for therapies directed against this lethal metastatic process.

BODY

Task 1: *To develop and optimize a novel polymersome-based CTC capture method using NIR-Eps bearing conjugated antibodies to EpCAM, N and O-cadherins and PSMA.*

We initially developed the anti-EpCAM NIR-EP in Year 1 as EpCAM forms the basis for the Cellsearch CTC capture method, the only FDA cleared CTC isolation and enumeration method and thus has proven prognostic importance in men with CRPC.[2,4] We were able to successfully construct an anti-EpCAM NIR-EP and tested this in cancer cells known to highly express EpCAM (T47D cells) and cells that lack EpCAM (PBMCs). As shown in **Figure 1**, these NIR-EPs exhibited excellent discriminatory abilities and sensitivity for EpCAM+ cells, with low non-specific binding to control cells. This discriminatory ability was noted with concentrations between 1.3–2.0 nM and different incubation periods for the cell lines (1 hour and overnight). We found that room temperature incubation provided the optimal temperature to maintain specific binding.

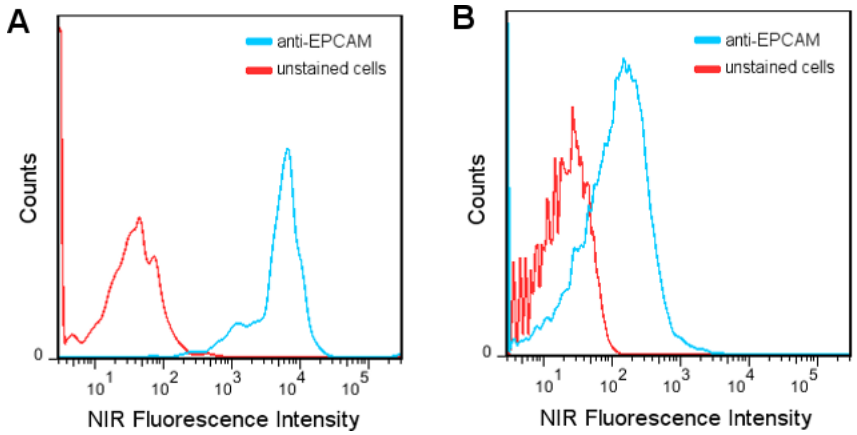


Figure 1 Flow sorting of EpCAM+ cancer cells (T47D cells, left) using the EpCAM-conjugated NIR-EP demonstrates clear signal separation vs. unstained cells using the Cy7 (>790 nm near infrared wavelength) channel. Peripheral blood mononuclear cells (PBMCs) that lack EpCAM demonstrate very little non-specific binding (right).

In later samples of EpCAM-targeted polymersomes however, batch-to-batch variability for binding to T47D cells was observed by flow cytometry. In some batches, high uptake of NIR-EPs per cell were observed; in others, uptake levels matched that of control NIR-EPs conjugated to an isotype-matched IgG antibody (**Figure 2**). This prompted us to reinvestigate our protocols in detail. Our previously used bicinchoninic assay (BCA) used to determine the degree of antibody functionalization on the surface on the NIREP was unable to distinguish between covalently bound-antibody and surface associated antibody. Therefore, the use of this assay to determine the efficiency of different antibody-coupling chemistries in some cases may have yielded inaccurate data.

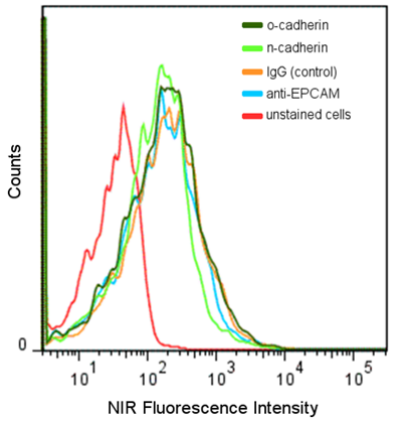
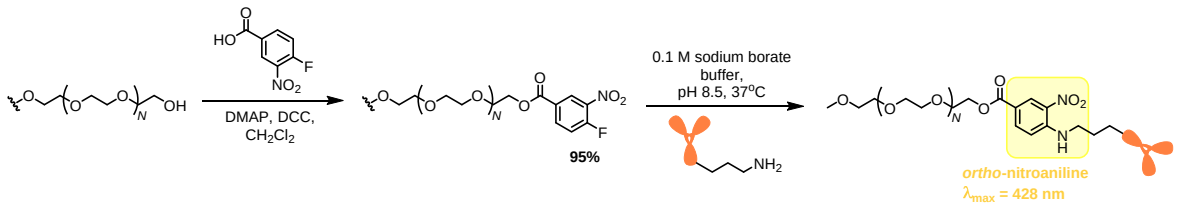


Figure 2 A later batch of anti-EpCAM NIR-EPs when incubated with EpCAM+ cells T47D showed no uptake relative to control NIR-EPs conjugated to an isotype-matched IgG antibody.

We have therefore invested time optimizing our coupling protocols and characterization methods as described below.

Conjugation chemistries

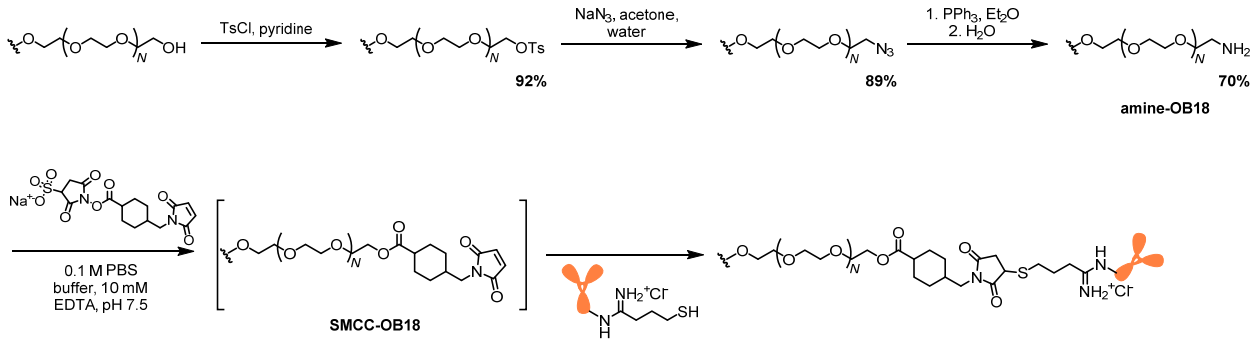
We have reexplored two orthogonal conjugation methods for the coupling of antibodies to the surface of NIREPs. The first method utilizes a fluoronitrobenzoic acid (FNB) functionality which is introduced onto the block-copolymer end hydroxyl group in a single, high yielding step (**Scheme 1**).[5]



Scheme 1 Functionalization of PDB-*b*-PEO diblock copolymer OB18 with FNB. Conjugation of a bioligand via a lysine residue yields a chromophoric *ortho*-nitroaniline linker highlighted in yellow.

The advantage of this method is twofold. Firstly, FNB is reactive to primary amines, which in the case of antibodies, is readily available *via* the protein N-terminus or surface lysine residues. The abundance of these residues in antibodies means prior chemical modification of antibodies is unnecessary, minimizing the possibility of antibody-deactivation through inadvertent modification of the complementarity determining regions (CDR). Secondly, conjugation of primary amines to the *para*-position of FNB yields a chromophoric *ortho*-nitroaniline, which absorbs strongly at 428 nm. This provides an internal 'reporter' that enables us to quantify covalently bound antibody using absorption at this wavelength. The Therien group has previously used FNB-based chemistry to successfully functionalize NIREPs with the cell-penetrating Tat peptide for tracking dendritic cells *in vivo*,[6,7] so we are confident we can adapt this protocol for the attachment of antibodies.

The second method we are exploring uses a sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) linker, which we can use to couple antibodies to the NIREP surface *via* a sulfhydryl group (**Scheme 2**).



Scheme 2 Synthesis of SMCC-functionalized PEO-*b*-PBD co-polymer OB18. SMCC-OB18 reacts with a thiol group introduced onto an antibody using 2-iminothiolane (Traut's reagent).

Sulfhydryl functionality can be introduced onto the surface of antibodies by modification of native lysines with 2-iminothiolane (Traut's reagent), without destruction of antibody immunoreactivity. Many examples of sulfo-SMCC being used as a protein-drug conjugate linker exist in the literature, most notably in the case of the 2013 FDA-approved chemotherapy drug ado-trastuzumab emtansine (Kadcyla®),where Herceptin is ligated through sulfo-SMCC to the cytotoxic agent mertansine (DM1).[8] We will evaluate the conjugation efficiency of antibodies to sulfo-SMCC functionalized NIREPs using size exclusion chromotography and SDS-PAGE.

NIREP construction

Using our newly validated functionalized OB18 polymer, we can construct NIREPs using our well-established methodology (**Figure 3**) to create monodisperse, unilamellar vesicles in the 100–200 nm diameter range (**Figure 4**).

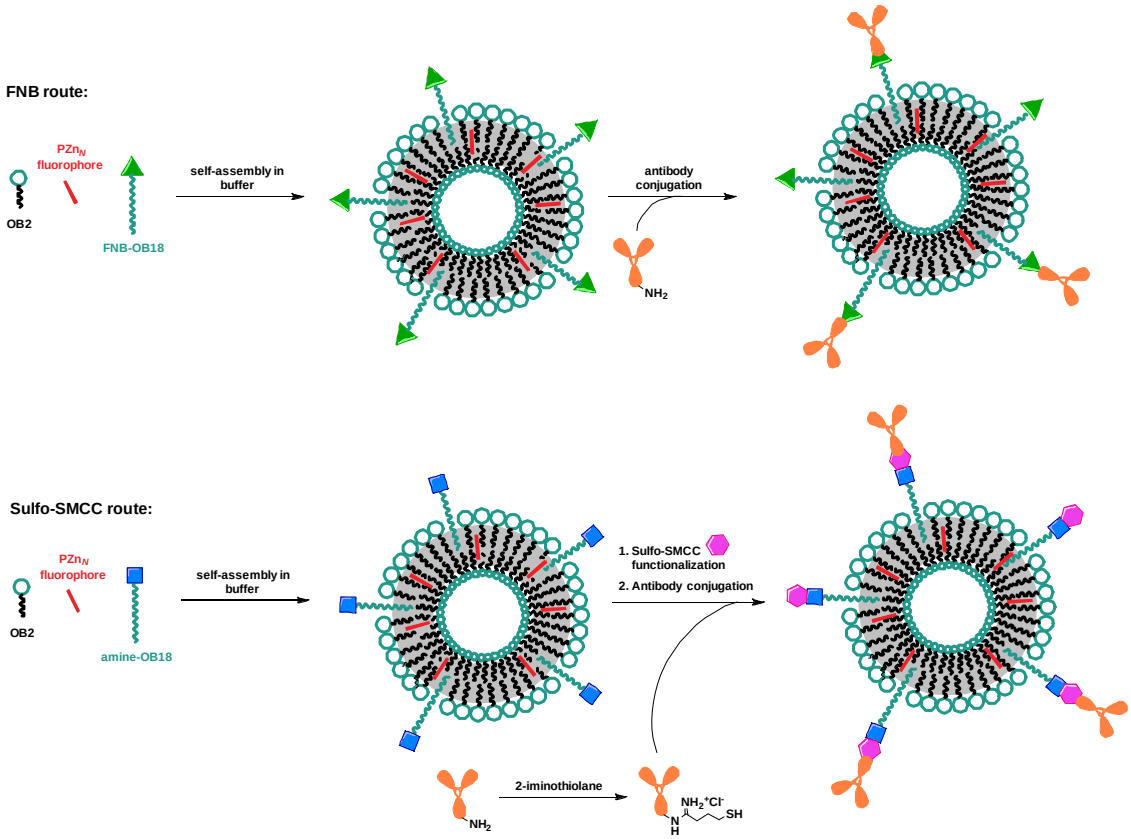


Figure 3 *Construction of antibody-conjugated NIREPs via FNB- and sulfo-SMCC routes.*

FNB/amine functionalized PEO(3600)-*b*-PBD(6800) diblock copolymer (OB18) was mixed with PEO(1300)-*b*-PBD(2500) diblock copolymer (OB2) in a 5:95 molar ratio. The mixed diblock co-polymer and porphyrin fluorophore (PZn_N) were dissolved in dichloromethane in a 40:1 polymer:fluorophore molar ratio. The solution was plated onto a roughened Teflon film and dried under vacuum overnight. Polymersomes were formed upon the addition of aqueous buffer (for FNB: 0.1 M sodium borate buffer, pH 8.5; for amine-OB18: 0.1 M PBS buffer, pH 7.5) and sonication for 1 h. A narrow size distribution of nano-size polymersomes was achieved with serial extrusion using a Liposofast Basic handheld extruder equipped with 400-, 200- and 100 nm polycarbonate membranes (Avestin Inc., Ottawa, Ontario). The resulting polymersomes are characterized by dynamic light scattering (DLS) and cryo-transmission electron microscopy.

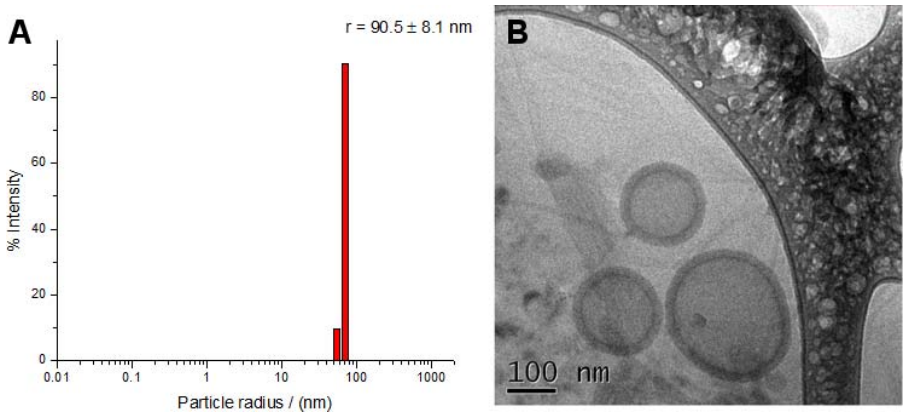


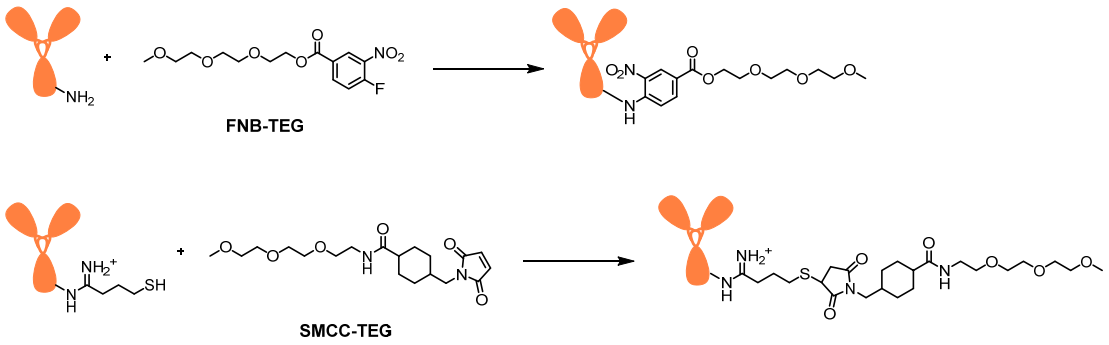
Figure 4 A. Dynamic light scattering data showing the distribution of polymersome sizes following extrusion. The resulting PEO-*b*-PBD polymersomes are highly monodisperse with diameters in the region of 181.0 ± 16.2 nm. *B*. Cryo-TEM image showing relatively monodisperse, unilamellar polymersomes in the 100–200 nm diameter size range.

To avoid maleimide deactivation by hydrolysis under aqueous conditions, SMCC-activated NIREPs are assembled using the precursor amine-OB18. The amine-NIREP can subsequently be treated with sulfo-SMCC, rapidly purified on a desalting column to remove excess linker, and then incubated with thiolated antibody. The presence of the cyclohexane ring in sulfo-SMCC confers significantly greater stability under aqueous conditions than our previously used *m*-maleimidobenzoyl-*N*-hydrosuccinimide ester (MBS) linker,[9] which will improve conjugation efficiencies of the antibody to the NIREP surface.

Antibody modification

Chemical modification of an antibody has the potential to adversely affect its immunoreactivity. The anti-EpCAM antibody contains many surface lysine residues which potentially could act as a chemical handle either for direct attachment to FNB-NIREPs, or for conversion to a SMCC-reactive thiol group. We will explore the effects of antibody functionalization in the following experiments:

1. Thiolation. We will assay the number of thiol groups introduced by 2-iminothiolane onto the surface of the antibody using Elmann's reagent. Reaction conditions (temperature, reaction time and reagent concentrations) will be altered accordingly to minimize the number of thiol groups introduced.
2. Coupling reactions. Anti-EpCAM antibody (AbD Serotec IgG1 clone VU-1D9, USA) will be conjugated to triethylene glycol (TEG) models as shown in **Scheme 3**. The immunoreactivity of the resulting conjugates will be evaluated using flow cytometry with T47D cells, and ELISA. If appropriate, different coupling conditions (temperature, buffer, reaction times, concentrations) will be explored.



Scheme 3 Reaction of antibodies with functionalized triethylene glycol (TEG) models to evaluate the effect of covalent modification on immunoreactivity.

Task 2: *Assessment of circulating tumor cell capture using novel antibody-targeted NIR-Eps in men with mCRPC.*

Given the delay in the formulation of NIR-EPs (see above), we were unable to test the antibody conjugated NIR-EPs in human subjects. We our dedicated to only going to human subjects testing for biomarkers that we have confidence in, in terms of sensitivity and specificity. We are confident that the modifications described above that we have performed in year 2 for task 1 will generate antibody conjugated NIR-EPs with greater sensitivity and specificity sufficient for testing in healthy volunteer blood and in patient samples, and in spiked samples of control cell lines in volunteer blood ex vivo. Thus, task 2 is awaiting a final chemistry product for each of the 4 NIR-EPs before we can enroll patients or volunteers onto our IRB-approved human subjects protocol. If one of the NIR-EPs demonstrates success on control cell lines, we will move forward with that NIR-EP first. As part of our 6 month no cost extension, we intend to complete the optimization process for task 1 that will enable human subjects testing and evaluation in control cells spiked into healthy volunteer blood. Results from task 2 will be described in the final report at the end of the no cost extension period.

KEY RESEARCH ACCOMPLISHMENTS—YEAR 2

The research accomplishment section should contain a brief summary of new findings or information that was obtained by performing the research outlined in the SOW.

- Revision of chemistry methods to permit an improved ability to conjugate antibodies to polymersomes with improved sensitivity and specifity
- Identification of a novel circulating tumor cell capture method to enable capture and characterization of a more mesenchymal CTC population defined by N-cadherin, OB-cadherin, or c-met expression. This method has led to the identification of rare cells clonally derived from epithelial prostate cancer cells that lack cytokeratin and express OB-cadherin and beta-catenin. Testing of the N-cadherin and c-met CTC capture method is ongoing.
- Receipt of a PCF Global Treatment Sciences Challenge Award (PI Armstrong) 2014-2016 based on preliminary data achieved with this DOD PRTA

REPORTABLE OUTCOMES FROM YEAR 2

1. Bitting RL, Schaeffer D, Somarelli JA, Garcia-Blanco MA, Armstrong AJ. The role of epithelial plasticity in prostate cancer dissemination and treatment resistance. Cancer Metastasis Rev 2014; Jan 11. See **Appendix 1**.
2. Awarded funding by the Prostate Cancer Foundation Global Treatment Sciences Challenge Award, July 2014 ($1.4 million).
3. Additional Grant proposals that arose from this award: DOD New Idea Award (funded 2012-2014), R01 (co-PI Garcia-Blanco and Armstrong), not funded: Alternative splicing and epithelial-mesenchymal plasticity in prostate tumors, submitted March 2013. R21 (PI Pei Zhong) for Tandem bubble-SAW technology for viable isolation and characterization of CTCs, start date 9/2014 (not funded).

CONCLUSIONS

While experimental setbacks we have experienced in the second year of our Department of Defense funding have delayed us in achieving the expected outcomes outlined in our Statement of Work, significant steps have been taken to optimize NIREP fabrication protocols. By ensuring that we can reproducibly conjugate immunoreactive antibodies to the surface of NIREPs, we can apply this methodology to rapidly fabricate a series of antibody-conjugated NIREPs for *ex vivo* detection and characterization of non-epithelial CTCs. Furthermore, our novel fabrication methodology itself will be useful to the wider scientific community. Our expectation is that upon optimizing our coupling procedures, we will quickly progress to a series of antibody-conjugated NIREPs targeted to EpCAM, N- and O-cadherin and PSMA. Our data with positive batches of anti-EpCAM-NIREPs demonstrate that NIREPs are a powerful tool for the optical detection of prostate cancer CTCs. We anticipate that a CTC technology that is able to identify and characterize a broad range of CTC phenotypes the differ by their gain or loss of epithelial character may be more broadly applicable than the currently approved Cellsearch test. For example, CTCs are often undetected in many patients with metastatic cancers, including prostate, breast, colorectal, pancreatic, and lung cancers, and EMT may explain this loss of marker expression and detection. Our method utilizes a sensitive approach with multiple antibodies that may bind to cell types of a range of phenotypes. We thus anticipate that the measurement of these cells will have greater clinical utility than existing assays and permit downstream molecular analyses once the cells are identified.

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Appendix 1. Manuscript on epithelial plasticity.

Cancer Metastasis Rev
DOI 10.1007/s10555-013-9483-z

The role of epithelial plasticity in prostate cancer dissemination and treatment resistance

Rhonda L. Bitting · Daneen Schaeffer · Jason A. Somarelli · Mariano A. Garcia-Blanco · Andrew J. Armstrong

Abstract Nearly 30,000 men die annually in the USA of prostate cancer, nearly uniformly from metastatic dissemination. Despite recent advances in hormonal, immunologic, bone-targeted, and cytotoxic chemotherapies, treatment resistance and further dissemination are inevitable in men with metastatic disease. Emerging data suggests that the phenomenon of epithelial plasticity, encompassing both reversible mesenchymal transitions and acquisition of stemness traits, may underlie this lethal biology of dissemination and treatment resistance. Understanding the molecular underpinnings of this cellular plasticity from preclinical models of prostate cancer and from biomarker studies of human metastatic prostate cancer has provided clues to novel therapeutic approaches that may delay or prevent metastatic disease and lethality over time. This review will discuss the preclinical and clinical evidence for epithelial plasticity in this rapidly changing field and relate this to clinical phenotype and resistance in prostate cancer while suggesting novel therapeutic approaches.

Keywords Epithelial plasticity · Prostate cancer · Metastasis · Epithelial–mesenchymal transition · Dissemination · Stem cell

R. L. Bitting · A. J. Armstrong (✉)
Division of Medical Oncology, Duke Cancer Institute, Duke University, DUMC Box 102002, Durham, NC 27710, USA
e-mail: Andrew.armstrong@duke.edu

R. L. Bitting · M. A. Garcia-Blanco · A. J. Armstrong
Department of Medicine, Duke University, Durham, NC, USA

R. L. Bitting · D. Schaeffer · J. A. Somarelli · M. A. Garcia-Blanco · A. J. Armstrong
Center for RNA Biology, Duke University, Durham, NC, USA

D. Schaeffer · J. A. Somarelli · M. A. Garcia-Blanco · A. J. Armstrong
Department of Molecular Genetics and Microbiology, Duke University, Durham, NC, USA

1 Introduction

In the USA, nearly 30,000 men die from prostate cancer (PC) each year, largely due to metastatic disease. Although the prognosis for patients with localized disease is good, for patients who develop metastatic disease, the 5-year survival rate is only approximately 30 % [1]. Androgen deprivation therapy (ADT) through either chemical or surgical castration is the first-line therapy for metastatic disease; however, response is temporary, and patients consistently progress to castration-resistant prostate cancer (CRPC), although at variable rates [2, 3]. The mechanisms underlying castration-resistant progression are likely diverse, but several key pathophysiological themes are emerging, including androgen receptor (AR) amplification, AR splice variants, and mutations in the ligand binding domain that render the AR constitutively active, as well as the induction of autocrine synthesis of androgen precursors within the PC itself [3–5]. In addition, key oncogenic drivers such as activation of the PI3K and Ras signaling pathways, loss of Rb and p53 function, and the emergence of epigenetic dysregulation and DNA repair defects underscore the complexity of advanced PC and the multifaceted genomic aberrations that promote treatment resistance.

Emerging from this genetic and epigenetic dysregulation is metastatic and hematogenous dissemination, frequently to bone, but also to other distant sites such as lung or liver. The clinical and pathological phenotype of lethal PC is quite heterogeneous, with autopsy studies demonstrating a high prevalence (>90 %) of bone metastases, and relatively high rates of visceral (liver, lung) metastases (>50 %)[6]. Histologically, metastatic PC is diverse, with some metastases exhibiting a neuroendocrine phenotype, others with poorly differentiated sheets of cells with or without spindle-like cells (sarcomatoid differentiation), and still others with a glandular well-differentiated epithelial appearance. Even

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within patients, phenotypic heterogeneity is commonly observed in histological appearance and protein and RNA biomarker expression, despite an underlying monoclonal metastatic genotype and epigenome [6–9]. These findings suggest substantial cellular plasticity at the level of RNA and protein expression within a given patient that is uncoupled from mutations and chromosomal anomalies. This metastatic dissemination leads to pathological fractures, anemia, bone marrow failure, fatigue, cachexia, progressive pain, and failure to thrive, hallmarks of the lethal clinical phenotype in advanced PC. While available hormonal, immunologic, and chemotherapeutic agents provide palliation and incremental improvements in survival, treatment resistance inevitably emerges over time, and thus, novel approaches are needed in this disease.

One potential approach to understanding metastatic PC and novel therapeutic strategies is through the study of epithelial plasticity (EP). EP describes the ability of a cell to undergo reversible phenotypic changes during invasion and dissemination. EP encompasses not only the epithelial to mesenchymal transition (EMT) during initial invasion and hematogenous dissemination and its converse of mesenchymal to epithelial transition (MET) during metastatic growth and colonization but also the more general concept of loss of the epithelial phenotype and replacement with a novel phenotype. While EMT is thought to confer upon the carcinoma cell the ability to invade and seed metastatic sites, MET is proposed to enable the disseminated cells to establish macrometastatic colonies. EP is emerging as a common theme in solid tumor pathobiology that encompasses both metastatic dissemination and treatment resistance, with links to underlying embryonal stemness and invasion programs [10]. EMT pathways are causally associated with the acquisition of stem-like properties (the ability to de-differentiate and self-renew) and may link tumor dissemination with phenotypic heterogeneity. Evidence to support EP in cancer biology is robust and has been established in both preclinical models of carcinoma and in patients with carcinomas [11–15]. Furthermore, EP biology has been linked to the risk of metastasis [10, 16]. In breast cancer models, for example, the induction of an EMT results in the expression of stem cell markers, increased metastatic potential, and resistance to conventional chemotherapy [10, 17–19]. Figure 1 depicts the general concept of EP during PC cellular dissemination. This review describing the role of EP in PC progression will start with a case discussion of secondary neuroendocrine differentiation of prostate cancer.

The concept of EP is illustrated in the following clinical vignette. Patient X is a 75-year-old African American man, with prostate-specific antigen (PSA) levels that were rising for many years, who presented in March of 2009 with an extremely elevated PSA of 50. He previously had two prostate biopsies that were negative for malignancy. His third prostate biopsy revealed Gleason 5+5=10 (high grade) adenocarcinoma with perineural invasion. Imaging revealed enlarged retroperitoneal lymph nodes up to 2 cm but no visceral or bony metastases. He was treated with combined androgen blockade, and PSA was undetectable within 9 months. Subsequent PSA and imaging progression was treated with sipuleucel-T immunotherapy followed by the novel androgen synthesis inhibitor abiraterone acetate, again with a good PSA response. However, after several months, rapidly enlarging lymph nodes in the setting of a stable PSA prompted a lymph node biopsy. The immunohistochemistry revealed strong staining for CD56 and synaptophysin with minimal PSA, prostatic acid phosphatase (PAP), or cytokeratin staining; together, these findings are suggestive of neuroendocrine differentiation. This neuroendocrine phenotypic transformation was not evident in his original prostate biopsy (Fig. 2). Evolving or secondary neuroendocrine transformation is increasingly recognized in advanced PC [20, 21] and may represent one form of EP similar to what has recently been described in lung cancer [22]. It is well documented from autopsy and pathology studies of human PC that many histological phenotypes emerge during hormonal therapy for PC, including squamous differentiation, neuroendocrine differentiation, and a general loss of markers of prostate differentiation [6, 23], as shown in Fig. 2.

Neuroendocrine differentiation (NED) occurs as one path to CRPC [24]. Although NED can arise *de novo*, it more commonly develops during hormonal therapy for PC [21]. NED does not have a strict clinical or pathological definition, but it is frequently defined histologically as the presence of neuroendocrine cells with chromogranin A or synaptophysin immunoreactivity. Chromogranin A also may be detectable in the plasma, where it correlates with the NED disease burden and is prognostic [20, 25]. The cells may also stain for synaptophysin or neuron-specific enolase, typically lack AR, and do not secrete PSA [26]. Clinically, NED is suspected when a patient has rapid disease progression, especially with visceral metastases, in the setting of a stable PSA. The presence of NED portends a poor prognosis, with frequent metastasis to the liver, transient response to chemotherapy, and survival often <1 year. While NED accounts for a large minority (perhaps 25 %) of aggressive CRPC [21], other mechanisms of EP leading to phenotypic changes are also likely to be important in human PC dissemination and treatment resistance.

This review focuses on the role of epithelial plasticity in the progression of prostate cancer, from both preclinical and clinical perspectives, and describes how EP may be associated with metastatic dissemination and treatment resistance. Additionally, we provide hypotheses and suggestions for therapeutic interventions to address EP in PC.

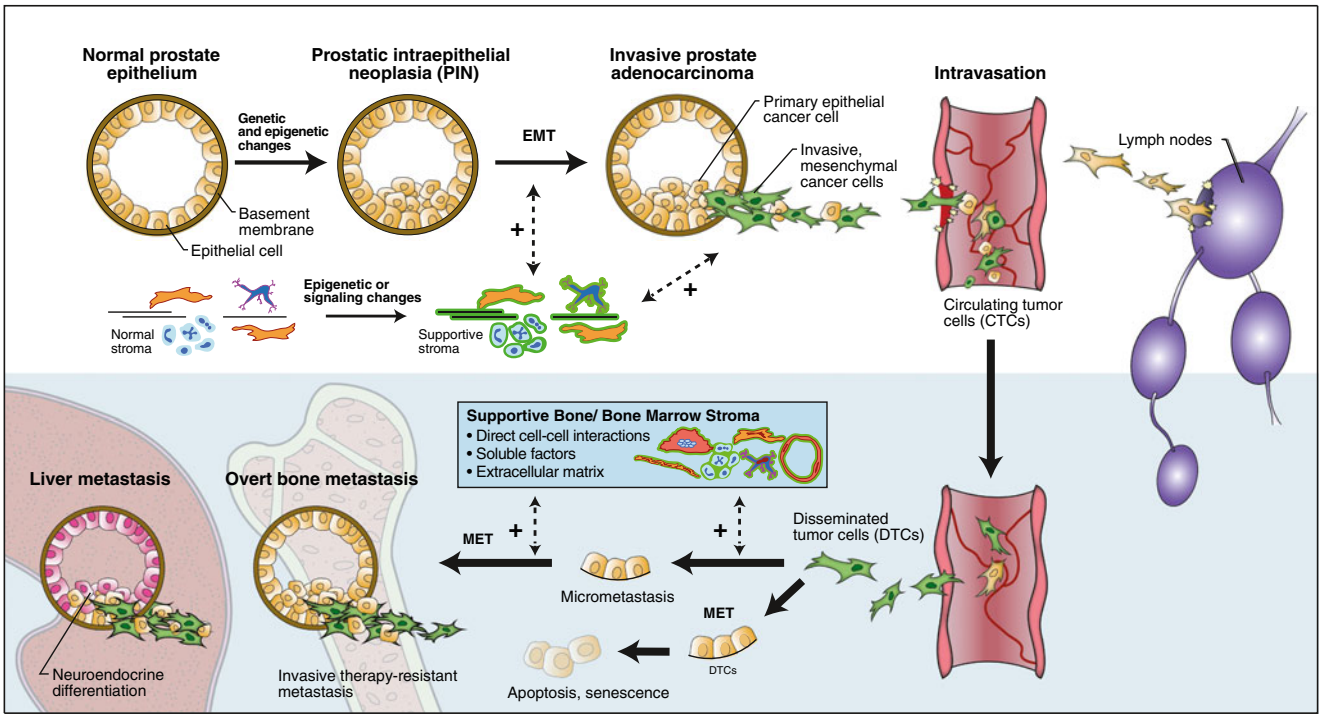


Fig. 1 Epithelial plasticity during prostate cancer dissemination. Due to genetic or epigenetic changes, normal prostate cells begin to grow uncontrollably, a premalignant process known as prostate intraepithelial neoplasia (*PIN*). In response to signaling from the surrounding stroma, some of these cells undergo an epithelial–mesenchymal transition (*EMT*) and invade through the basement membrane. These invasive cells enter the bloodstream and may exist as epithelial circulating tumor cells (*CTCs*), mesenchymal CTCs, or CTCs with a dual phenotype. Upon exiting the vasculature, disseminated tumor cells (*DTCs*) may sit dormant or undergo apoptosis. Other DTCs undergo a mesenchymal-epithelial transition (*MET*) and grow as detectable macrometastases. In PC, bone metastases are typical and are initially AR dependent, progressing through a range of AR mutations or splice variants, and other oncogenic and tumor suppressor mutations. Visceral metastases are atypical, are variably AR dependent, and generally involve loss of an epithelial phenotype and are enriched for a neuroendocrine or anaplastic phenotype. EP is not clearly linked to the process of lymph node metastasis; instead, nodal metastases likely involve other forms of invasion or migration

2 Preclinical evidence of EP in PC

EP in epithelial-origin tumors (carcinomas) involves the reversible loss or reduction of epithelial biomarkers [e.g., E-cadherin, zona-occludens (ZO)-1, cytokeratin isoforms, fibroblast growth factor receptor-2 (FGFR2) isoforms, and miR-200 family] and the loss of differentiation antigens [27]. In PC, these differentiation antigens include PSA, PAP, and prostate specific membrane antigen, among others. Epithelial markers may be replaced by mesenchymal markers and transcription factors such as SNAIL, Slug, TWIST1, ZEB1/2, and others, and/or increased expression of stemness pathways, such as Hedgehog or NOTCH signaling. While NED is relatively common in PC progression, it may occur as a result of EP, a fixed evolution through novel mutations, or perhaps both [21, 28]. Suggesting the importance of plasticity, however, in lung cancer a change to a neuroendocrine-like phenotype can occur in response to treatment and is reversible when treatment is stopped [22]. Also implying the relevance of EP in dissemination and disease progression, at autopsy, many PC patients demonstrate histologic heterogeneity, in which multiple phenotypes are evident despite an underlying clonally derived tumor, as shown in Fig. 2.

EMT and MET are highly dynamic and mediated by multiple proteins, microRNAs, and second messengers, including but not limited to those involved in transcription, posttranscriptional gene regulation, signal transduction, cytoskeletal remodeling, migration, invasion, and proliferation. Given the inherent complexity in such a system, it is likely that many incomplete or partial EP-like events take place in different contexts. One such example of an EP-like event is the mesenchymal to amoeboid transition, in which mesenchymal cells are able to alter their cellular shapes to pass through the basement membrane without degrading it [29].

Another type of EP is osteomimicry, in which PC cells can acquire bone-like properties [30]. PC most commonly metastasizes to bones, and the ability of PC cells to mimic the bone environment may enable them to survive and colonize in this new environment. The upregulation of β2-microglobulin, an immune regulator protein, can induce EMT, promote osteomimicry, and lead to bone metastasis in mouse models of prostate and other cancers [31]. Furthermore, PC cell lines

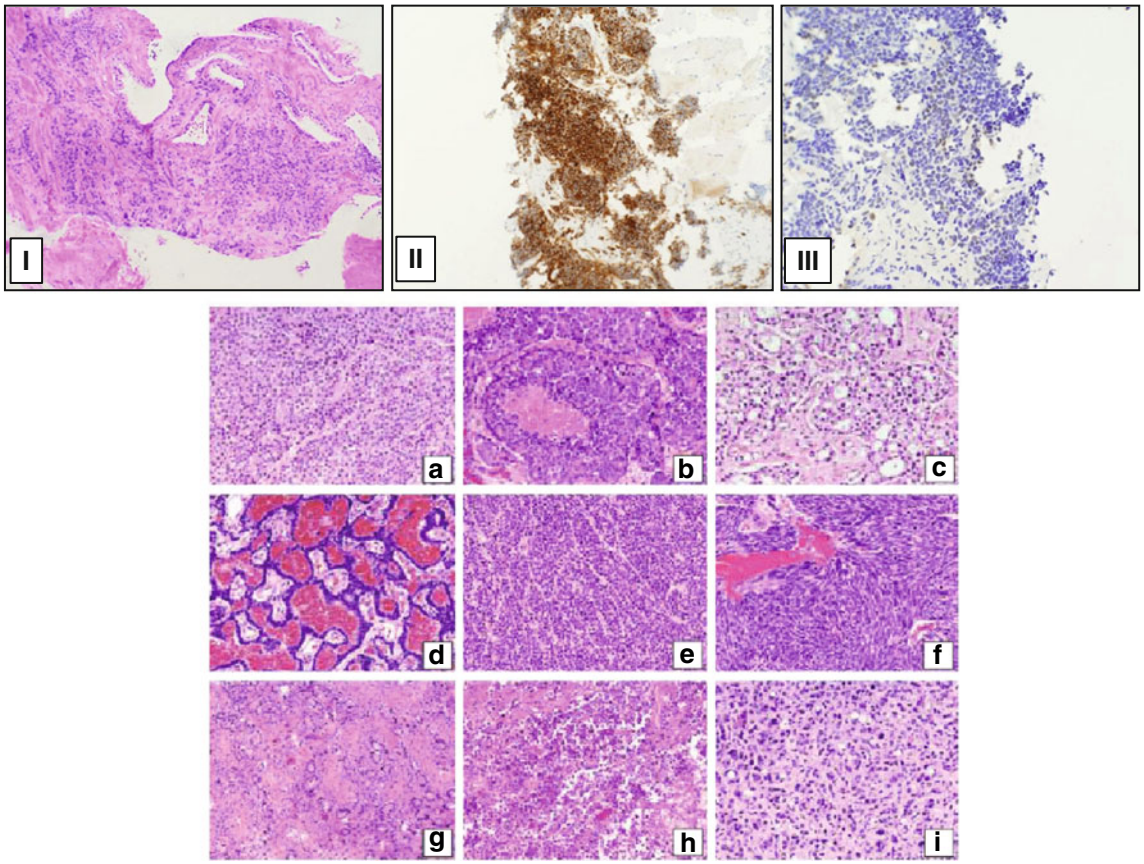


Fig. 2 Examples of prostate cancer phenotypic transformations that emerge with treatment. The *top panel* is illustrates the phenotypic changes that arise during treatment of patient X, as described in the clinical vignette. His initial prostate biopsy showed high-grade prostate adenocarcinoma (*I*), but neuroendocrine differentiation emerged as his disease progressed, illustrated by strong synaptophysin (*II*) with weak PSA staining (*III*). All images are at ×100 magnification. The *bottom panel* shows the histological spectrum noted at autopsy of treated prostate cancer. **a**–**c** Variations of Gleason grade 4 and 5 adenocarcinoma. **d**, **e** Neuroendocrine differentiation. **f** Small cell carcinoma. **g** Well-differentiated Gleason grade 3 disease. **h** Undifferentiated growth pattern. **i** Signet ring differentiation. (Figure reprinted with permission from the American Association for Cancer Research: Rajal Shah et al. [6], p. 9211.)

can be forced to differentiate into osteoblast-like cells or adipose cells [32], suggesting that PC cells have the inherent capability to change phenotypes. Additional studies have established that PC cells produce soluble factors that lead to the expression of osteoblast-specific genes [33]. We have identified osteoblast (OB)-cadherin frequently in the circulating tumor cells (CTCs) of men with CRPC, illustrating the clinical relevance of this form of phenotypic change [15]. If the process of osteomimicry could be effectively targeted therapeutically, metastasis of PC to bone could potentially be prevented.

A variety of pathways and biomarkers have been confirmed to be associated with EP in cell lines and preclinical xenograft or genetically engineered models of PC; a smaller subset has been validated in human PC progression. Table 1 provides an overview of those pathways and biomarkers linked, preclinically and clinically, to EP in PC. In PC cell lines, EMT can be induced or may occur spontaneously. ARCaP cells, for example, were derived from a patient with metastatic CRPC and gave rise to stable epithelial, $ARCaP_E$, and mesenchymal, $ARCaP_M$, sublines [34]. Other mesenchymal sublines have been generated from a parental epithelial PC line, including derived EPT1 lines, generated by *in vitro* passaging of the EP156T cell line [35] and the PZ-HPV-7T subline, generated by subrenal capsule xenografting of the PZ-HPV-7 cells [36]. PC-3 and DU145 cells additionally commonly express a range of mesenchymal and epithelial phenotypes [37]. These cell lines are valuable tools for studying EP in PC in the laboratory setting and provide further evidence for EP in clinical settings. The following sections discuss transcriptional activators or repressors of EMT/MET, signaling pathways, microenvironmental cues, microRNA regulators, stemness pathways, and other regulators of phenotypic change and the role that each play in promoting EP and dissemination in PC.

2.1 Transcriptional activation of EP

Several transcription factors have been shown to be sufficient for inducing EMT in carcinoma cell lines by repressing the E-

Table 1 Selected biomarkers and pathways associated with EP in preclinical models and patients with PC

Pathway and biomarker associated with EP in PC	Link to stemness	Link to AR signaling in PC	Validation in human PC	References
EMT-related transcription factors				
SNAIL	N	N	N	[38, 39, 41, 42]
TWIST1	Y	N	Y	[45–51]
Id-1	N	N	N	[56–61]
Slug/Snai2	N	Y	Y	[42, 43]
ZEB1/2	N	Y	N	[44, 228]
ETS-family (ERG)	N	Y	Y	[225, 227–232]
HIF-1α	N	N	Y	[125–127]
Cell surface protein expression				
Loss of E-cadherin	Y	N	Y	[27]
N-Cadherin	Y	N	Y	[235]
OB-Cadherin	N	N	Y	[15]
EGFR	N	N	N	[109]
FGFR1	N	N	Y	[116, 122]
FGFR2 isoforms	Y	N	N	[115–117]
Stemness pathways				
Hedgehog/NOTCH-1	Y	N	Y	[173, 222]
WNT/β-catenin	Y	Y	Y	[73–78]
NANOG	Y	N	N	[123]
BMI	Y	N	N	[199]
TGF-β signaling				
SMAD4	N	N	Y	[97]
TGF-β RIII	N	N	Y	[94]
COUP-TFII	N	N	Y	[98]
BMPs	Y	N	N	[99]
Intracellular protein signaling				
AR	N	Y	Y	[16, 64–67]
PTEN/PI3K pathway	Y	Y	Y	[68, 69]
DAB2IP	Y	Y	Y	[79–81]
EZH2	Y	Y	Y	[80, 166]
Ras pathway	Y	Y	Y	[69, 71]
NF-κB pathway (IL-6/8)	Y	Y	Y	[82–87]
Micro-RNA species				
miR-200 family	Y	N	N	[172, 174, 177]
Chaperone proteins				
HSP27	N	Y	Y	[14, 108]

cadherin promoter; however, only a few of these transcription factors, including SNAIL, Slug, ZEB1, TWIST1, and Id-1 have been identified as having a role in EMT during PC progression. SNAIL is a zinc finger transcription factor that has been shown to induce EMT in many types of human cancers, including breast [38] and colorectal [39]. Forced expression of SNAIL in epithelial PC lines $ARCaP_E$ and LNCaP is sufficient to induce at least a partial EMT, as evidenced by altered biomarker expression and migration. In contrast, SNAIL inhibition in mesenchymal PC-3 cells induces epithelial biomarker expression [40]. Consequently, expression of SNAIL is thought to be both necessary and sufficient to induce EMT, but the relationship of SNAIL to human PC remains to be established. Of note, SNAIL expression also induces a neuroendocrine phenotype in PC cells [41], suggesting that SNAIL expression may play promote differentiation into several cell states. Another zinc-finger transcription factor required for the initiation of EMT in PC cells is Snai2, commonly known as Slug. Knockdown of Slug in PC-3 cells results in increased expression of E-cadherin, suggesting that Slug is required for maintenance of the mesenchymal phenotype [42]. Importantly, Slug acts as a

coactivator of AR and, in androgen-deprived conditions, provides a growth advantage to PC cells [43]. ZEB1 is another zinc-finger transcription factor that is both necessary and sufficient to induce EMT in PC [44].

TWIST1, a basic helix loop helix (bHLH) transcription factor, has been most widely studied in EMT in breast cancer [45] but has also been shown to induce EMT in gastric [46] and head and neck cancers [47], and is clinically associated with distant metastasis and poor prognosis in these tumor types [48–50]. In PC cell lines, knockdown of TWIST1 has been shown to induce a partial MET with an increase in E-cadherin expression, highlighting the importance of TWIST1 in maintaining a mesenchymal phenotype [51]. Further supporting the role of TWIST1 in EMT is the observation that epigenetic regulation of the TWIST1 promoter is needed for a common p53 mutant to induce EMT. Wild-type p53 is a transcription factor that, when activated by cellular stress, promotes cell cycle arrest and apoptosis [52, 53]. Mutations in p53 are common in cancer cells, are responsible for the functional loss of the tumor suppressor, and may result in downregulation of the epigenetic regulator BMI-1 and resultant upregulation of TWIST1 expression [54]. Dysregulation of p53 is common in metastatic PC, and loss of p53 function may promote EMT through TWIST1 deregulation, or through a separate pathway involving microRNA deregulation [55].

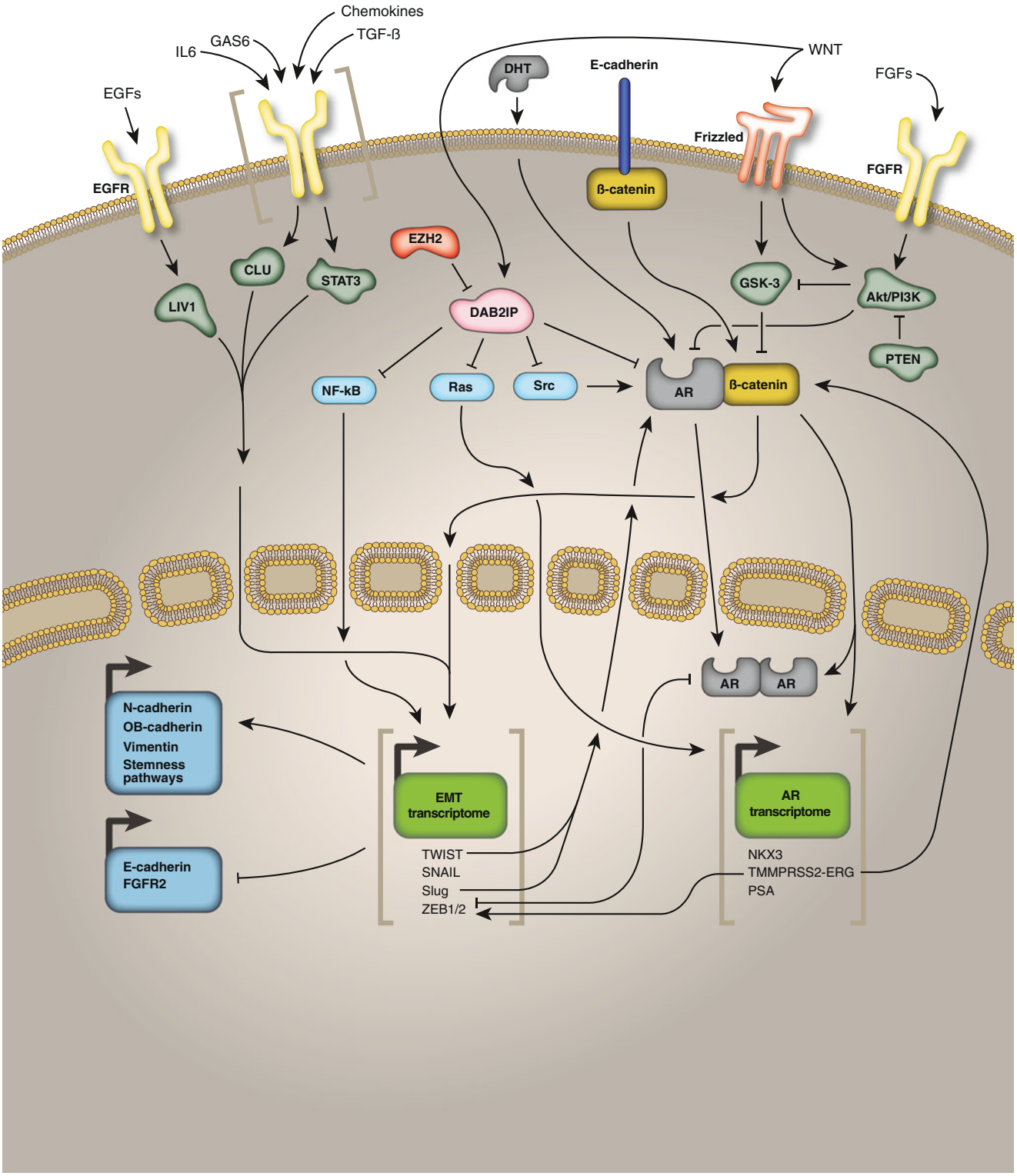
Inhibitor of differentiation/DNA binding (Id-1) is another bHLH transcription factor that has a dominant negative effect on other bHLH transcription factors because it lacks a DNA binding domain. Id-1 is involved in several physiological processes, including inhibition of differentiation and delayed senescence [56], and is upregulated in several carcinomas including prostate [57]. Id-1 interacts with caveolin-1 (Cav-1) [58], which is a membrane protein involved in signaling transduction and is upregulated in metastatic PC [59, 60]. Combined expression of ID-1 and Cav-1 induces cell migration and EMT in LNCaP and PC-3 cells. Specifically, the interaction of Id-1 and Cav-1 induces Akt activation, which is thought to be the mechanism of EMT induction [58]. Cav-1 promotes Akt activation by repressing the activity of a serine/threonine protein phosphatase, PP2A [61], and suppression of PP2A requires Cav-1 binding to PP2A [58]. Together, these results suggest that the interaction between Id-1 and Cav-1 activates Akt and subsequent EMT. Further work in human PC is needed to decipher the relationship between the Id-1 pathway and dissemination/differentiation. Interestingly, NED in human PC has been linked to deregulated PI3K/Akt/mTOR signaling, raising the possibility of a link between EP, the ID-1, and PI3K pathways, and phenotypic transformation [62, 63]. In summary, a range of transcription factors have been linked in PC cell lines and model systems to EMT and invasion and are typically accompanied by alterations in other cellular pathways important in cellular differentiation, survival, and DNA repair.

2.2 Signaling in EP

In preclinical models of PC, transcriptional activation of EP can be induced via a wide range of signaling pathways. Both intracellular activators and soluble growth factors can mediate phenotypic plasticity, and extensive crosstalk between multiple signaling pathways illustrates the importance of redundancy and feedback loops in regulating cellular survival, dissemination, and plasticity. See Table 1 for a select listing of the roles of these pathways in PC progression. In addition, Fig. 3 depicts key signaling nodes in PC that regulate EP.

AR signaling is required for normal development of the prostate [16] and is a common target for therapeutic intervention in PC. The AR pathway is activated by 5α-dihydrotestosterone (DHT), a metabolite of testosterone, and binding of DHT to AR initiates translocation of the nucleus, where AR acts as a transcription factor to transcribe genes involved in cell cycle progression [64]. Importantly, androgens can also modulate EMT in some PC cell lines. For example, treatment of PC-3 and LNCaP cells with DHT leads to downregulation of E-cadherin and upregulation of N-cadherin and SNAIL [16]. Furthermore, knockdown of AR in LNCaP and CWR22 cells sensitizes cells to androgen-mediated EMT [16], suggesting that AR may protect PC cells from undergoing EMT in the presence of androgens, whereas AR inhibition may promote EMT. In normal mouse prostate tissue and LuCaP35 xenografts, ADT induces EMT and stemness features [65]. In LNCaP cells, AR represses ZEB1 expression and vice versa [65], indicating that a feedback loop

Fig. 3 Key signaling nodes in prostate cancer that regulate epithelial plasticity. This is a simplified and broad schematic describing the interplay of EP signaling and transcription with AR in aggressive PC. Signaling through multiple and interacting pathways leads to EMT through a variety of mechanisms. Signaling by EGFs, IL6, GAS6, chemokines, and TGF-β, through their respective receptors, can lead to increased expression of EMT transcription factors (TFs). EMT TFs, including but not limited to TWIST, SNAIL, Slug, and ZEB1/2, can then upregulate mesenchymal biomarker expression (e.g., N-cadherin, vimentin, OB-cadherin) and downregulate E-cadherin expression. TWIST also inhibits FGFR2 expression. These TFs can interact with AR in varying ways. For example, TWIST and Slug can activate AR, while ZEB 1 and AR are reciprocal inhibitors of each other. AR also upregulates NKX3-1, which in turn represses TWIST. When Wnt ligands are present, β-catenin moves to the nucleus and activates target genes linked to EMT and survival. β-Catenin can also act as a cofactor with AR. DAB2IP negatively regulates Ras and NF-κB signaling and, when epigenetically silenced by EZH2, leads to EMT and PC metastasis through activation of the Ras and NF-κB pathways. Loss of DAB2IP also activates AR through phosphorylation by Src kinase and β-catenin pathways. AR activation can lead to increased TMPRSS2-ERG fusion, which in turn can activate EMT through ZEB1/2 and increase β-catenin signaling. FGFs signal through the PI3K/Akt pathway to promote tumor proliferation, and the PI3K/Akt pathway also negatively regulates AR. DHT is the AR ligand, and when available to tumor cells, also promotes growth. Note that not all pathways discussed in the manuscript are shown in this figure



between these two proteins may exist. AR also upregulates NKX3-1, which represses TWIST1 via binding to the TWIST1 promoter [66]. Contrary to the above findings, which suggest that AR inhibits EMT, ectopic expression of AR in BPH-1 cells induces EMT, whereas knockdown of AR downregulates EMT markers [67], suggesting that AR may play a different role in culture conditions than within the tumor microenvironment. The connections between AR signaling and EP are likely complex and context dependent, and many signaling pathways including β-catenin, Src kinase,

Akt/mTOR, and G-protein receptors can signal directly to AR independent of ligand, further adding to the complexity. Tables 1 and 2 provide an overview of these associations.

Loss of PTEN, a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and member of the Akt signaling pathway, is observed in approximately 40 % of human PC [68]. Genetic knockout of PTEN in mouse models mirrors the clinical disease course but without progression to metastatic disease [69]. To identify the additional components required for metastatic disease progression, an analysis of human PC microarrays revealed that the Ras pathway is significantly upregulated in both primary and metastatic PC tissue [69]. Interestingly, a prostate-specific Ras/PTEN-null mouse model results in PC, followed by EMT and metastasis in 100 % of mice. Models with PTEN null or Ras mutant tumors alone do not develop macrometastases, suggesting the importance of cooperative signaling in the promotion of dissemination [69]. PTEN loss is linked to the acquisition of stemness properties and loss of a differentiated phenotype in PC model systems [69, 70]. Given that aberrations in the PTEN/PI3K, AR, and Ras signaling pathways are present in nearly 100 % of metastatic PC [71], it is likely that drivers of EP are associated with these three key oncogenic pathways in CRPC.

The wingless/int1 (Wnt) gene was originally identified as a retroviral oncogene and a modulator of embryonic development in *Drosophila melanogaster* [72]. Decades later, it was shown that anomalous activation of the Wnt/β-catenin pathway is a driver of multiple human cancers, including prostate [73]. The Wnt pathway is activated by the binding of Wnt ligands to their receptors. When Wnt ligands are present, β-catenin moves to the nucleus and activates target genes linked to EMT, invasion, proliferation, and survival [74]. In PC, β-catenin may act as cofactor with AR [75], and increased β-catenin expression and change in localization have been observed in advanced disease [76, 77]. Another member of the Wnt family, Wnt5a, mediates EMT via activation of the membrane type I matrix metalloproteinase (MT1-MMP), which is a membrane-bound MMP involved in degrading the extracellular matrix, and is upregulated in breast and prostate cancers [78].

Also involved in the Wnt pathway, DAB2IP, a Ras GTPase-activating protein, has been shown to possess tumor suppressive properties via maintenance of an epithelial phenotype [79]. Knockdown of DAB2IP leads to EMT in PC-3 cells, while overexpression of DAB2IP decreases mesenchymal biomarker expression and migratory potential of PC cells via antagonism of the Wnt/β-catenin pathway. Moreover, knockdown of DAB2IP in PC-3 cells leads to increased metastatic burden in a xenograft mouse model [79]. Importantly, DAP2IP levels positively correlate with E-cadherin and negatively correlate with vimentin in primary tumor tissue from PC patients [79], which supports the role of the Wnt pathway in mediating PC progression via regulation of EP. Epigenetic loss of DAB2IP has been linked to EMT and PC metastasis through overexpression of the epigenetic regulator EZH2 and subsequent downstream activation of nuclear facto kappa B (NF-κB) and Ras pathways [80]. Furthermore, the loss of DAB2IP is linked to enhanced AR activation and AR variant activity through phosphorylation by Src kinase and β-catenin pathways, providing a novel

Table 2 Selected clinical states of PC and evidence of associations with EP as a treatment resistance mechanism

Clinical Disease State of PC	Description of EP Association with Outcome	Direct evidence from men with PC	References
Localized disease			
Surgery (radical prostatectomy)	E/N cadherin switch associated with PSA recurrence, metastasis after surgery	Y	[223]
	Loss of CK or PSA expression, increased TWIST or vimentin in localized disease correlates with outcomes	Y	[221, 224]
Radiation therapy	Induction of WNT16B in stroma mediates radioresistance in PC	N	[150]
Active Surveillance	ERG overexpression in biopsy specimens associated with progression during surveillance	Y	[229]
PSA recurrent disease			
Androgen deprivation therapy	ADT induction of EMT transcription factors	Y	[65]
Metastatic PC			
Immunotherapy	Immunotherapy against epithelial targets leads to mesenchymal tumor escape	N	[240]
Docetaxel chemotherapy	Loss of CK, overexpression of stemness pathways (NOTCH/Hedgehog) in docetaxel-treated metastases, PC cell lines	Y	[200]
Cytotoxic DNA-damaging agents	Induction of DNA-stress response in stroma leads to WNT16b induction and EMT, treatment resistance to mitoxantrone	Y	[150]
Circulating tumor cell expression	Common expression of vimentin, N-cadherin, CD133, OB-cadherin in CTCs from men with metastatic CRPC	Y	[15]

link between EMT, dissemination, and AR signaling mediated through the epigenetic and thus reversible loss of DAB2IP [81].

NF-κB transcription factors regulate a variety of immune and inflammatory responses and developmental processes (reviewed by [82]). Levels of NF-κB correlate with prognosis in PC patients, and increased NF-κB signaling correlates with disease progression in a subset of PC patients [83]. NF-κB regulates EMT by directly or indirectly upregulating multiple EMT transcription factors, the mesenchymal intermediate filament protein vimentin, and matrix metalloproteases MMP2 and MMP9 [84]. In addition, IkappaB kinase alpha activation by receptor activator of NF-κB ligand (RANKL) inhibits expression of the Maspin protein and metastatic dissemination. Maspin is a serpin family member, expression levels are inversely correlated with metastatic potential in human PC, and its signaling or epigenetic regulation may be causally related to dissemination [85]. In PC cell lines, induction of EMT leads to upregulation of RANKL [86]. Interestingly, RANKL activation results in osteoclastogenesis *in vitro* [86], suggesting that upregulation of RANKL via EMT induction may promote skeletal metastasis. NF-κB also mediates EMT via downregulation of fibulin and activation of CXCL1/GROα [87], a chemokine that promotes angiogenesis and enhances cancer cell proliferation [88]. These examples highlight the complexity of signaling networks that may cooperate to drive EMT and the metastatic cascade in advanced disease.

One of the best-studied initiators of EMT is the transforming growth factor beta (TGF-β) family of cytokines and their receptors, TGF-β RI, II, and III. TGF-β can induce EMT, as evidenced by increased expression of mesenchymal biomarkers in multiple PC cell lines [89]. Importantly, TGF-β can induce EMT in an androgen-independent cell line, PC-3, and in an androgen-dependent line, LNCaP, suggesting that the ability of TGF-β to induce EMT is independent of AR expression [90]. TGF-β treatment also induces clusterin (Clu) expression during EMT, with Clu functioning as a molecular chaperone to protect against cellular stresses [91]. Clu is transcriptionally activated by TWIST1, and this activation is required for TGF-β-induced EMT [89]. Clu has emerged as an important therapeutic target in men with CRPC, and given its role in mediating chemotherapy resistance, its link to EP may be equally important [92, 93]. In addition, loss of TGF-β RIII is common in human PC, through deletions or epigenetic dysregulation, and this is accompanied by enhanced invasion and relapse after surgery [94]. The paradox of TGF-β signaling in human PC, in which there is increased TGF-β expression and tumor suppression early in the disease, followed by tumor promotion during disease progression, may be explained through altered intracellular signaling. Specifically, TGF-β signaling may initially promote invasiveness and escape from the primary tumor microenvironment; however, loss of TGF-β in distant metastasis may promote an epithelial phenotype and ultimately colonization [95, 96]. For example, loss of SMAD4 is consistently identified in metastatic as compared to localized PC, indicating that loss of this tumor suppressor may facilitate dissemination [97]. Importantly, SMAD4 was identified as a component of a four-gene signature, along with PTEN, cyclin D1, and SPP1, that is prognostic of biochemical recurrence and metastatic disease in human PC [97]. It has recently been shown that COUP transcription factor II (COUP-TFII) regulates SMAD4-dependent transcription in PTEN-null tumors, making a TGF-β dependent checkpoint ineffective and leading to EMT and metastasis [98]. Taken together, loss of SMAD4 signaling and altered TGF-β signaling is associated with the acquisition of an invasive phenotype and metastatic dissemination in PC. Finally, a TGF-β superfamily member, bone morphogenetic protein-7, protects against bone metastases in PC through the induction of epithelial differentiation [99], possibly by counteracting SMAD family members. However, the role of BMPs and TGF-β signaling in general in mediating EP and PC dissemination remains an area of active investigation.

The role of the interleukin-6 (IL-6)/STAT3 pathway, which activates inflammatory responses during infection and oncogenesis [100, 101], in EMT has been demonstrated in head and neck [102], nonsmall cell lung [103], and breast cancers [104]. This pathway may also be important in PC, as IL-6 can induce EMT in some PC cell lines. Importantly, induction of EMT by IL-6 requires Hsp27 expression. Specifically, knockdown of Hsp27, an ATP-independent molecular chaperone that is induced in response to stress [105–107], reverses the pro-EMT effect of IL-6. The role of Hsp27 in IL-6-induced EMT is likely through the transcriptional activation of TWIST1. Hsp27 expression is required for TWIST1 expression upon treatment with IL-6, and transcriptional activation is mediated by direct binding of STAT3 to the TWIST1 promoter [108]. Taken together, this suggests that Hsp27 is needed for IL-6-induced EMT but also can act independently to induce EMT. IL-6 has also been linked to activated stromal and immune cell cross-talk and induction of EP/stemness in PC [14], suggesting a complex relationship between the tumor microenvironment and EP.

Other pathways implicated in PC progression preclinically through an EP mechanism include the following: (1) the epidermal growth factor receptor (EGFR) pathway via expression of LIV-1, a zinc transporter [109]; (2) macrophage inhibitory cytokine-1, a member of the TGF-β superfamily that plays a key role in regulating growth and differentiation in response to stress [110–112]; (3) β2-microglobulin mediation of the hemochromatosis protein, a member of the nonclassical major histocompatibility complex signaling pathway [31]; (4) the kallikrein family of serine proteases, which induce EMT and invasiveness [113]; and (5) ubiquitin C-terminal hydrolase-L1,

UCH-L1, a deubiquitinating enzyme, the expression of which is both necessary and sufficient to induce EMT [114].

2.3 Alternative splicing in EP

There is evidence that fibroblast growth factor receptor (FGFR) signaling plays a role in PC onset and progression. The FGFRs are a family of four receptor tyrosine kinases (FGFR1-4) that bind to a family of fibroblast growth factors (FGFs) (reviewed in [115]). Binding of FGFs induces dimerization of the receptors and signaling via MAPK and PI3K/Akt pathways. FGFR1-3 transcripts are alternatively spliced within their ligand binding domains to give IIIb and IIIc isoforms. The IIIb and IIIc isoforms are typically expressed exclusively in epithelial and mesenchymal cells, respectively [115]. A switch from FGFR2-IIIb to FGFR2–IIIc in nontumorigenic rat prostate epithelial cells leads to malignancy [116]. A subset of human PC specimens expresses the IIIc isoform, although metastatic samples predominantly express the epithelial IIIb isoform [117]. Work from our group and others has identified several splicing factors that regulate FGFR2 isoform switching, including PTBP1 [118], RBFOX2 [119], and ESRP1 [120]. Interestingly, RBFOX2 and ESRP1 have also been implicated in mediating numerous splicing events that help to maintain mesenchymal or epithelial phenotypes, respectively, in breast cancer cell lines [121]. It is possible that these splicing factors may play a role in EP during PC by inducing FGFR2 isoform switching and by regulating the splicing of a number of other EP-related transcripts.

Both FGFs and FGFRs are known to be upregulated in PC, including FGFs 1, 2, 6, 7, 8, and 9 and FGFR1 [115], and inducible expression of FGFR1 leads to adenocarcinoma and EMT in a mouse model of PC [122]. FGFR1-induced adenocarcinomas show loss of the epithelial-specific IIIb isoform, increases in Sox9, MMP15, and genes related to TGF-β signaling, and metastases to the liver and lymph [122]. The lack of validated FGFR isoform-specific antibodies has impaired the translation of these findings in human PC, and this work is ongoing.

2.4 Microenvironmental cues as mediators of EP

The effect of hypoxia within the tumor microenvironment on EP has been widely studied in human cancer. Hypoxia is capable of inducing EMT in PC-3 and LNCaP cells, as evidenced by a switch to a more mesenchymal morphology and increase in mesenchymal biomarker expression [90]. Additionally, PC-3 cells grown under hypoxic conditions have an increased migratory and invasive phenotype. Hypoxia also induces transcripts associated with stemness, including Nanog and EZH2 in PC-3 and LNCaP cells [123].

The molecular mechanism of hypoxia-induced EMT is through the stabilization of HIF-1α, a transcription factor expressed at low oxygen concentrations [124]. Importantly, PC-3 cells grown under hypoxic conditions have increased HIF-1α protein expression [123] and forced expression of HIF-1α in LNCaP cells results in a partial EMT, as evidenced by a decrease in E-cadherin expression and an increase in vimentin [125, 126]. In addition, in other models systems, HIF-1α expression increases TWIST1 transcription to promote EMT and metastasis [127, 128]. To date, however, regulation of TWIST1 by HIF-1α in PC has not yet been studied.

Hypoxia also plays an indirect role in the initiation of the EMT cascade by stabilizing the Axl/GAS6 axis. Axl is a receptor tyrosine kinase that induces cell survival/proliferation upon binding its ligand, GAS6. The Axl/GAS6 pathway is important for metastasis of several carcinomas [129–133], and is adversely prognostic [134–138]. Axl is necessary for EMT, as evidenced by reduction in mesenchymal biomarkers and increased migration and invasion upon knockdown of Axl in PC cells [139]. GAS6 downregulates expression of its receptor, Axl, and hypoxia is sufficient to prevent GAS6-mediated downregulation of Axl. Therefore, hypoxia acts to stabilize Axl/GAS6 signaling, which ultimately results in induction of EMT [139].

Another mechanism by which the tumor microenvironment can contribute to EP is by fibroblasts in the host stroma, which secrete soluble factors, such as growth factors and extracellular matrix [140, 141]. Activated fibroblasts (AFs) are necessary for the growth and differentiation of PC cells [142, 143], and AF can induce EMT. Specifically, prostate-specific fibroblasts isolated from men with benign hyperplasia and can be activated by either TGF-β treatment or by exposure to conditioned media from PC-3 cells to induce EMT. EMT induction in PC-3 cells also promotes stemness, as evidenced by an increase in prostasphere formation, an increase in CD133 positive cells, and an increase in the percentage of $CD44^{high}/CD24^{low}$ cells [14]. Furthermore, induction of EMT in PC-3 cells activates the COX-2 pathway and HIF1A, both of which are involved in the inflammatory response. Upon knockdown of COX-2 and HIF1A in PC-3 cells, EMT cannot be induced, suggesting that the proinflammatory axis is required for initiation of EMT. In addition to initiating an inflammatory response, induction of EMT in PC-3 cells also results in reactive oxygen species (ROS) production. With inhibition of ROS production by treatment with antioxidants, prostate AF can no longer induce EMT, stemness, or the inflammatory response pathway [144]. Together, these data suggest that prostate AF produce ROS and activate the proinflammatory response to induce EMT and stemness [14, 144].

The generation of ROS has been associated with EMT in several model systems, including human ovarian carcinoma

cells [145], renal tubular epithelium [146], and mammary epithelial cells [147]. In the context of PC, there are conflicting reports about the role of ROS in mediating EP. For example, ROS increase during SNAIL-induced EMT, and a ROS scavenger, *N*-acetyl cysteine, causes a partial reversion of EMT [148]. On the contrary, psoralidin, a natural pro-oxidant chemical, induces ROS production, but leads to downregulation of β-catenin and Slug, upregulation of E-cadherin, and inhibition of migration and invasion in PC cell lines [149]. While it remains unclear whether ROS stimulates or prevents EMT, it is possible that different ROS levels can have variable effects on the phenotypic status of a cell. For example, moderate ROS can induce cell proliferation, but higher levels lead to apoptosis ([149] and references therein). In addition to hypoxia and ROS, stromal cells can induce EMT through a range of soluble mediators such as chemokines and the soluble protein WNT16B. DNA damage from radiation or chemotherapy can to induce WNT16B and promote EMT in the neighboring prostate epithelial cells, leading to invasion and treatment resistance [150]. Furthermore, activated fibroblasts and other stromal cells such as fat cells or bone marrow derived cells may be recruited into the prostate from distant sites to promote EP [151]. Thus, a number of microenvironmental and host insults can promote EP, dissemination, and treatment resistance in PC. In addition to microenvironmental drivers of EMT, there is also evidence that MET in metastatic colonization may be mediated by the microenvironment. For example, DU-145 cells re-express E-cadherin upon coculture with human hepatocytes, and re-expression of E-cadherin also leads to chemoresistance, suggesting that MET may serve a protective role against chemotherapeutics at metastatic sites [152]. Similarly, coculture of DU-145 and PC-3 cells with primary rat hepatocytes leads to re-expression of E-cadherin and cytokeratin and reduced levels of vimentin [153], and coculture of $ARCaP_M$ cells with bone marrow stromal cells results in re-expression of E-cadherin in the $ARCaP_M$ cells [154], lending further support for the idea that microenvironmental cues at the sites of metastatic dissemination may lead to MET. Using a reporter of MET based on alternative splicing of a mesenchymal IIIc exon of FGFR2, clusters of MET can be identified within AT3 Dunning rat mesenchymal prostate tumors [155]. These regions of MET also express E-cadherin and ZO-1 and localize to areas rich in collagen, suggesting that the interaction of tumor cells with collagen or some other microenvironmental driver may contribute to MET.

2.5 Epigenetics in EP

Histone deacetylase inhibitors (HDACI) have been studied as potential cancer therapeutic agents based on the increased expression and activity of HDACs in carcinomas (as reviewed in [156]). When evaluating the efficacy of HDACI in PC cell lines, the cells unexpectedly undergo EMT upon treatment with both suberoylanilide (SAHA) and trichostatin A (TSA), as evidenced by a more mesenchymal morphology, upregulation of ZEB1 and vimentin, and increased stemness and migration. The mechanism by which HDACI induce EMT is thought to be via hyperacetylation of EMT promoters, which create a more relaxed chromatin state to promote transcription. Specifically, PC-3 cells treated with TSA and SAHA have an increased amount of acetylated histone 3 associated with the vimentin, ZEB2, and slug promoters, which results in increased EMT signatures [157]. These findings may explain the limited single agent activity of HDACIs in the clinic as therapy for CRPC and suggests that combination approaches are needed [158].

Despite the limited utility of HDACI in clinical treatment of PC, there is evidence for the importance of epigenetic changes in PC. For example, multiple myeloma SET domain (MMSET), a histone methyltransferase that is associated with the dimethylation of histone H3 lysine 36, a mark of active transcription [159], can be upregulated in PC [160], with high expression associated with PC recurrence [161]. Overexpression of MMSET in PC cells leads to increased expression of mesenchymal biomarkers and a more migratory and invasive phenotype. Conversely, knockdown of MMET in PC cells leads to decreased migration and invasion. MMSET promotes EMT by binding the TWIST1 promoter and increasing TWIST1 transcription, suggesting that MMSET epigenetically regulates TWIST1 to induce EMT [162].

SIRT1 is another histone deacetylase, which is implicated in the stress response [163] and apoptosis [164] and induces EMT in PC cells. Moreover, knockdown of SIRT1 in PC cells induces MET. ZEB1 is required for SIRT1 to induce EMT, as ZEB1 recruits SIRT1 to the E-cadherin promoter for deacetylation of histone H3, which suppresses E-cadherin transcription. This suggests that ZEB1 interacts with SIRT1 to downregulate the E-cadherin promoter to induce EMT [165]. Likewise, enhancer of zeste homolog 2 (EZH2), which is involved in gene silencing by histone methylation, is overexpressed in advanced PC and can mediate the silencing of E-cadherin [166]. Interestingly, a survey of primary PC samples and metastatic bone biopsies showed that 70 % of primary PC samples have a methylated E-cadherin promoter with heterogeneous E-cadherin expression, while 87 % of metastatic bone biopsies contain an unmethylated E-cadherin promoter with homogenous E-cadherin expression [167]. Together, these results demonstrate that EMT can be epigenetically regulated and provide a mechanism linking EMT with PC progression.

2.6 MicroRNAs in EP

MicroRNAs (miRs) are important regulators of gene expression that play diverse roles in development, metabolism, and

the pathogenesis of cancer (as reviewed in [168, 169]). Several miRs have been shown to regulate EP, including miR-21, miR-31, miR-29a, miR-135, and the miR-200 family (reviewed in [170]). In PC, ectopic expression of miR-1 or miR-200 precursors reduces Slug-dependent EMT, restores E-cadherin expression, and significantly reduces the invasive potential of PC-3 cells [171]. Similarly, PC-3 cells overexpressing platelet derived growth factor D undergo EMT, which leads to reduced levels of miR-200 family members [172]. Re-expression of miR-200b induces MET [172] and represses NOTCH1, a driver of stemness [173]. Taken together, these data suggest that miR-200b acts as a tumor suppressor at least partially through regulation of NOTCH1 expression.

Loss of the ZEB1 and ZEB2 repressors, miR-200c and miR-205, has been shown in docetaxel resistant PC-3 and DU145 lines and re-expression of either miR led to E-cadherin upregulation [174]. This suggests that loss of these miRs during docetaxel-mediated EMT may contribute to chemotherapeutic resistance. Additional studies have shown that expression of miR-182, miR-203, and miR-29b in mesenchymal prostate cells can induce MET [175]. While many miRNAs have been associated with MET, miR-21 has been shown to induce EMT in RWPE-1 cells [176] and is the only mesenchymal-specific miRNA currently identified in human PC.

Although there are a number of *in vitro* studies on miRs in PC, few studies have investigated levels of EP-related miRs in PC specimens. While both miR-200c and miR-29 contribute to an epithelial phenotype *in vitro*, the correlation between these miRNAs and clinical outcome is less clear. For example, the epithelial specific miRNAs, miR-200c and miR-29b, are upregulated in men with CRPC compared to those with localized disease [177] and in patient-matched normal tissue compared to PC [178]. This is inconsistent given that an epithelial-specific miRNA is associated with both metastasis and healthy prostate tissue. Similarly, the mesenchymal-specific miRNA miR-21 is higher in CRPC tissues compared to localized PC [177]. One possible explanation is that an epithelial phenotype can be simultaneously associated with normal prostate tissue and also be needed for metastatic colonization via MET. It is conceivable that the mesenchymal miR-21 is associated with an early metastatic event, while miR-200c is associated with a late metastatic event that requires MET for colonization. Further complicating the relationship between miRs, EP, and clinical outcome, the loss of epithelial-specific miR-205 reduces time to biochemical recurrence in human PC [179].

2.7 Dietary and small molecule control of EP in PC

A number of dietary substances and small molecules can induce epithelial differentiation (MET) and possibly invasion in PC cell lines. One of the most frequently cited supplements is silibinin, the active compound in milk thistle extract, which has shown some promise as a regulator of EP. Silibinin treatment inhibits growth of PC cell lines [180], synergizes with various chemotherapeutic compounds to induce apoptosis [181–185], and attenuates AR signaling by inhibiting AR nuclear localization [186]. Furthermore, silibinin also mediates MET in PC cells, as evidenced by reduced proliferation, adhesion, and migratory potential of PC cell lines [187], decreased expression of mesenchymal biomarkers [188], and upregulation of the epithelial biomarkers [189]. HIF-1α, which induces EMT in response to hypoxia via upregulation of TWIST1 [128], is also inhibited by silibinin [190].

Dietary consumption of another compound, Genistein, an isoflavone found in soy beans, is associated with a lower risk of PC and PC metastasis ([191] and references therein). Interestingly, Genistein treatment results in MET of PC cells, as evidenced by altered biomarker expression and decreased invasion [191]. Exposure of Genistein also reduces CD44+ cancer stem cells, inhibits the Hedgehog-Gli1 pathway [192], and upregulates miR-574-3p, which decreases proliferation, migration, and invasion of PC cells [193]. Pathway analysis indicates that miR-574-3p controls several genes involved in the Jak-STAT and Wnt signaling networks [193]. This suggests that a small molecule, Genistein, controls EP via miR-mediated regulation of the Wnt and other signaling pathways.

Treatment with the proteasome inhibitor salinosporamide A (NPI-0052) also causes an MET in the mesenchymal-like DU-145 cells, with reduced levels of SNAIL and upregulation of E-cadherin. SNAIL repression is driven by inhibition of NF-κB and upregulation of Raf kinase inhibitory protein (RKIP), a known inhibitor of metastasis [194]. RKIP expression in DU145 cells leads to reduced levels of SNAIL expression, whereas SNAIL overexpression in LNCaP cells antagonizes RKIP levels, leading to increased metastatic capacity. Moreover, treatment with a specific NF-κB inhibitor, dehydroxymethylepoxyquinomicin, mirrors the EMT repression that is observed upon treatment with salinosporamide A [194]. Together, these results implicate the proteasome as a potential modulator of EMT via a SNAIL/NF-κB/RKIP pathway.

2.8 Stemness as a mediator of EP

Work by the Weinberg laboratory and other groups has shown that EMT results in enrichment of cell populations with stem cell properties of self-renewal, clonogenic growth, and ability for differentiation in several cancer models [10, 195, 196]. In PC, CD44+ LNCaP and DU145 cells lose E-cadherin and are more invasive than their CD44- counterparts [197]. EMT has also been associated with the acquisition of a stem-like phenotype in PC-3 and $ARCaP_M$ cells [173]. Similarly, knockdown of the ETS transcription factor ESE/EHF in immortalized prostate epithelial cells leads to EMT, acquisition of stem-

like properties, tumorigenic capability, and metastatic dissemination [198]. Association of cancer-associated fibroblasts with PC-3 cells also leads to EMT, along with upregulation of CD133 and an increase in $CD44^{high}/CD24^{low}$ cells, which display self-renewal capacity and tumorigenicity [14]. In PC model systems, overexpression of the polycomb repressor BMI-1 is required for de-differentiation, prostate stem cell renewal, and has been linked to malignant transformation [199]. In the clinical context, evidence for EP and stemness can be found in the examination of CTCs from men with CRPC. CTCs have been found to coexpress epithelial and mesenchymal markers, and >80 % of CTCs from CRPC patients also express the stemness marker CD133, suggesting that stemness may play a role in modulating EP during metastatic dissemination through the vasculature [15]. Finally, evidence is strong for the loss of epithelial biomarker expression during castration and chemotherapy-resistant progression in human PC, and this EP is linked to upregulation of stemness pathways including Hedgehog and NOTCH signaling, suggesting the importance of the dual regulation of EP by these embryonic programs [200].

There is, however, also evidence of PC cell lines in which cancer stem cells are enriched for an epithelial phenotype. E-Cadherin positive subpopulations of DU145 and PC-3 cells express embryonic stem cell markers SOX2, OCT3/4, Nanog, and c-Myc. Furthermore, the E-cadherin positive populations form tumors, while E-cadherin negative sublines do not [201]. Additionally, DU145 cells treated with chemotherapy generates drug-tolerant lines with low tumor initiating capacity, and addition of 5′-aza-deoxycytidine to drug-tolerant cells leads to re-expression of E-cadherin and CD44+, with increased tumorigenic potential [202]. Moreover, it has been shown that an epithelial-like subpopulation of PC-3 cells is enriched in tumor initiating cells (TICs) while the mesenchymal subpopulation are depleted in TICs [37]. Overexpression of SNAIL in the epithelial-like TICs reduces their self-renewal and metastatic capacity, concomitant with an EMT-like event [37]. Conversely, combined knockdown of SNAIL, ZEB1, and TWIST leads to an epithelial phenotype, enhanced spheroid formation, and self-renewal programs [37]. In a review of CSCs in PC, a distinction is made between TICs and CSCs, highlighting that the existence of TICs suggests the clonality of tumor cells rather than a hierarchical structure of the tumor [203]. Yet, despite this distinction, the data surrounding CSCs and EP highlight the dynamic and complex relationships between stem-like programs and EP pathways and suggest that EMT may not be the sole driver of PC cell tumorigenicity and invasive potential.

Based on these findings, we hypothesize that it is the ability to interconvert reversibly between epithelial and nonepithelial stem-like phenotypic states (plasticity) that drives metastatic spread and lethality in PC (and likely other solid tumors), rather than the epithelial or mesenchymal state in isolation.

3 Evidence of EP in treatment-resistant and disseminated PC

The above sections suggest a role for EP in the development of invasiveness, treatment resistance, and dissemination in PC model systems. Observing this plasticity in the clinic is a greater challenge given that EP is transient, may occur in rare cellular populations at the invasive edges of the tumor, and that the gold-standard biomarkers of EP in PC are still being defined. Furthermore, metastatic tissue in PC is not collected or analyzed routinely, metastatic tissue architecture and phenotype can be heterogeneous, and the ability to observe EP biomarkers in patients is likely context dependent. EP is linked to drug resistance [204], and there is emerging evidence that EP mediates resistance to local therapy (surgery or radiation), hormonal therapies, immunotherapies, and chemotherapeutics commonly used to treat PC. The following sections detail the clinical evidence to supporting a causal relationship between EP and treatment failure due to resistance in human PC. Selected clinical states of PC and their associations with EP are highlighted in Table 2.

3.1 Detecting EP in PC

One of the challenges in establishing the existence and relevance of EP in PC metastasis is the difficulty visualizing the process. To establish distant metastases, invasive cancer cells likely circulate in the bloodstream and settle in other organs, which in CRPC is often bone. Evidence supporting EP is found through an analysis of circulating tumor cells (CTCs). CELLSEARCH® (Janssen/Veridex) is the only FDA-cleared technology for the detection of CTCs, which are defined as nucleated, cytokeratin-positive, and CD45-negative cells immunomagnetically captured from the bloodstream using antibodies against epithelial cell adhesion molecule [205]. CTCs can be enumerated to provide prognostic information in multiple tumor types [206–209], but more importantly, CTCs carry genotypic and phenotypic information about an individual's tumor at a discrete point in time. A substantial number (30-40 %) of men with advanced metastatic CRPC do not have detectable CTCs using the CELLSEARCH® epithelial-based method [210], and recent evidence indicates that there is phenotypic heterogeneity among CTCs, with some CTCs expressing not only epithelial proteins but also mesenchymal and stemness proteins, indicators of EP [15]. We have found that a range of EP biomarkers are expressed in CRPC CTCs, including loss of E-cadherin and gain of N-cadherin, vimentin, CD133, β-catenin, and OB-cadherin. Importantly, many CTCs have a dual epithelial and mesenchymal/stemness phenotype, suggesting the importance of this duality in treatment resistance and dissemination [15]. This EP biology is not unique to PC, as variable phenotypes have been observed in CTCs from other malignancies, such as

lung [211, 212], colorectal [213], and breast cancer [214], suggesting a broad conceptual parallel. Therefore, EP may explain the underdetection of CTCs in patients with advanced malignancy using the standard epithelial antigen-based technology [15, 215, 216]. There are a number of technologies under development that employ nonepithelial targets for CTC capture and characterization and may provide a noninvasive window into the role of EP in cancer metastasis [217].

Given its dynamic and transient nature, visualizing EP is a major challenge radiographically. EP may be routinely seen but not clinically recognized through tumor imaging. In PC, there is well-documented discordance between PSA measurements and imaging responses. For example, technetium-99 bone scans indirectly assess osteoblastic activity induced by PC metastases to bone, and the interpretation is often complicated by the "flare phenomenon," which is an osteoblastic reaction that may occur in response to treatment where new or increased intensity of existing lesions is noted. The flare gives the appearance of worsening of bony metastatic disease, but is not adversely prognostic. For instance, in a phase II study of abiraterone plus prednisone in patients with metastatic CRPC, over half of the patients responding to abiraterone by PSA criteria had initial worsening of the bone scan, but more than 80 % of those scans improved subsequently, consistent with the flare phenomena [218]. We hypothesize that this initial flare may represent an element of EP induced by treatment, in which PC osteomimicry linked to induction of EMT becomes evident during the initial phases of treatment. During treatment-induced EMT, the mesenchymal, stem-like cells mimic osteoblasts and take up more technetium-99, accounting for these early changes on bone scans. Although this imaging flare temporarily stabilizes and often improves, the bone lesions typically progress at a later time point, indicating persistent viable tumor in these regions of bone scan activity. Given that a number of agents used to treat men with PC, such as hormonal therapies, can induce this reaction, and that osteomimicry markers may likewise emerge during ADT [15, 65], the bone scan flare may be imaging evidence of a shift toward a bone-forming mesenchymal state and thus plasticity.

In contrast to the flare phenomenon described above, in a phase II study of the c-met/VEGFR2 inhibitor cabozantinib in metastatic CRPC, nearly 80 % of patients had complete or partial resolution of bone scan lesions after 12 weeks of therapy, but bone scan response did not correlate with PSA or CTC response [219]. The initial imaging improvement with cabozantinib is typically short-lived, with the re-emergence of active bone lesions over time in the same regions, indicating persistent viable tumor despite the disappearance on scans. We hypothesize that the changes visualized on bone scan during the course of treatment with cabozantinib may be the result of cellular plasticity and induction of MET. This induced MET would shift away from the osteoblastic mesenchymal state in bone metastases and toward a more epithelial, nonbone-forming state, and lead to a transient reduction in technetium-99 uptake. This may be accompanied by a rise in PSA due to this epithelial differentiation driven by AR activity [220], which is often disconnected from the radiographic changes. Thus, PSA changes reflecting epithelial biology and bone scan changes representing mesenchymal tumor biology may be clinical biomarkers of EP. Further studies to quantify these changes in the context of tumor biopsies during a range of therapies are needed.

3.2 EP in localized PC

Although advanced metastatic PC is known to be a heterogeneous disease [6], it has been demonstrated that most metastases arise from a single precursor lesion in the primary tumor, suggesting that lethal PC has a monoclonal origin [8]. Therefore, differences in phenotype rather than genotype must account for the heterogeneity, and even in localized PC, there is evidence for EP. For example, TWIST1 is absent in benign prostatic tissue but expressed in prostate adenocarcinoma cells, and higher levels of TWIST1 expression are associated with higher Gleason scores in the primary tumor [221]. By immunohistochemistry, higher expression of EMT markers can be seen at the invasive front of the tumor versus the center of the tumor. For example, E-cadherin expression decreases at the invasive front while vimentin and ZEB-1 expression increase [222]. Similarly, in the primary prostate tumor, the combination of weak E-cadherin and strong N-cadherin expression, or high vimentin or TWIST1 expression, predict early dissemination and clinical recurrence [223, 224].

A frequently observed genetic lesion in human PC is the TMPRSS2-ERG fusion, in which exon 1 of TMPRSS2, an androgen-regulated serine protease, is joined to exons 4–9 of the ERG gene, an erythroblast transformation-specific (ETS) transcription factor [225]. The fusion protein TMPRSS2-ERG is present in more than half of all PC [226]. Interestingly, TMPRSS2-ERG fusion can induce EMT via activation of the Wnt/β-catenin pathway [227]. In addition, EMT can be induced *in vitro* and *in vivo* by overexpression of the TMPRSS2–ERG fusion. Here, EMT is mediated by ZEB1 and ZEB2, and chromatin immunoprecipitation assays revealed that TMPRSS2–ERG directly binds the ZEB1 promoter [228]. This suggests that the TMPRSS2-ERG fusion may be associated with more aggressive disease by controlling ZEB1-induced EMT and offers a biological explanation for the prognostic significance attributed to detection of the TMPRSS2-ERG protein. In a cohort of men with localized PC undergoing active surveillance, those men with the TMPRSS2-ERG fusion had a higher likelihood of PC-specific mortality [229]. Additional studies show that the presence of the fusion protein predicts for recurrence after prostatectomy [230] and portends a worse survival [231].

This is controversial, however, as a recent metaanalysis found no association between ERG overexpression via TMPRSS2–ERG fusion and recurrence or mortality [232], and the relevance of the genomic rearrangement may be variant dependent. For example, one variant found in approximately 5 % of PC is the TMPRSS2-ERG fusion together with the deletion of sequences 5′ to ERG, and the presence of this variant confers a poor prognosis [231].

Radiation therapy is commonly used to treat localized PC and, in many men, is curative; however, greater than one third of men with high-risk disease will relapse after local radiotherapy. There is concern, however, that the emerging tumor clones in men who fail radiotherapy may undergo EMT and develop an associated treatment resistance. For example, ionizing radiation induces DNA double-strand breaks, and the DNA damage response can induce stromal cells to secrete WNT16B, a soluble protein that may induce EMT mediated through the NF-κB pathway in neighboring PC cells. WNT16B overexpression has been observed during cytotoxic chemotherapy and radiation in PC patients and model systems and has been recently linked to treatment failure and dissemination [150]. Thus, EP is emerging as an adaptive stress-activated mechanism of resistance to radiotherapy and cytotoxic therapy that is induced by stromal signaling.

3.3 EP in metastatic PC

Gene expression analysis of single CTCs revealed increased expression of EMT-related genes in CRPC patients compared to castrate-sensitive patients, suggesting that activation of EMT-related genes may be associated with disease progression [233]. For example, NOTCH-1, which has been associated with an EMT and stem cell phenotype [173], is significantly upregulated in bone metastasis compared with the primary prostate tumors, suggesting that NOTCH-1 may be important for PC progression [222].

As discussed above, EP is increasingly recognized as a mechanism underlying drug resistance, and in PC, evidence exists for the upregulation of mesenchymal biomarkers during androgen deprivation in cell lines, animal models, and in patient tumor specimens. For example, expression of the mesenchymal marker N-cadherin increases after androgen deprivation in men treated prior to surgery [234]. Furthermore, ADT has been shown to induce an EMT, possibly by removing the inhibitory effect that AR signaling has on the transcription factor, ZEB-1. However, these cells are able to revert to an epithelial phenotype upon replacement of testosterone, indicating EP [65]. N-Cadherin expression is rare in untreated PC, increases with androgen deprivation, and is highest in the castration-resistant setting [235]. In the primary prostate tumor, the combination of weak E-cadherin and strong N-cadherin expression predicts for early biochemical failure and clinical recurrence [223]. N-Cadherin expression has been associated with a more rapid progression to castration resistance, which may be circumvented preclinically through direct targeting with monoclonal antibodies to N-cadherin [235]. With this rationale, one could hypothesize that high N-cadherin expression would predict for resistance to agents that block AR signaling; however, clinical studies are needed to confirm the role of mesenchymal biomarkers in predicting treatment resistance to pathways that target androgen synthesis or signaling.

Metastatic sites may variably express EP markers, and this variability may exist within and between patients. For example, in a metastatic survey study of human PC, lymph node metastases frequently had lower E-cadherin expression levels than bone metastases in the same patient [236]. This heterogeneity may reflect different modes of invasion or migration, such as collective sheet migration to lymph nodes, which may be independent of EP, as compared to a TGF-β-mediated hematogenous dissemination that has a greater requirement for EMT/MET [237]. In PC, metastatic site has prognostic importance, as lymph node metastatic CRPC has the most favorable prognosis, followed by bone-metastatic and visceral metastatic CRPC [238].

Docetaxel, an antimitotic microtubule-stabilizing agent, is the most commonly used chemotherapy for PC, and resistance to this agent often emerges within 6–12 months of treatment initiation. Recent evidence shows that PC cells lacking the epithelial marker cytokeratin (CK18 and CK19) are able to survive treatment with docetaxel. These docetaxel-resistant cells are more abundant in metastatic sites as compared to the primary tumor [200]. In cell line and xenograft models, docetaxel-resistant cells are induced by activation of stemness pathways important for EP and can be depleted by combining docetaxel with agents that target the NOTCH and Hedgehog signaling pathways [200]. Loss of CK or PSA in prostatectomy specimens is associated with recurrence and metastasis as well [239], suggesting that identification of cytokeratin- or PSA-negative PC cells may predict for resistance to local or systemic therapies, but additional validation is necessary. Given that taxanes have been shown to induce EP and stemness in several model systems, accompanied by treatment resistance and dissemination, therapies that reduce this resistance mechanism are needed [204].

EP may also lead to resistance to immunotherapy. Treatment with an epithelial-based complementary DNA (cDNA) vaccine results in regression of prostate tumors in mice, but when resistant tumors eventually emerge, these tumors lack PSA expression and gain mesenchymal markers. Revaccination with a cDNA library derived from the resistant tumors eradicates the tumors and cures the mice. Reversal of the vaccination strategy, giving the mesenchymal vaccine followed by the epithelial vaccine, is ineffective [240]. This is further evidence for the role of EP in treatment resistance and may provide clues as to how to tailor treatment to target these resistance pathways.

Given that the FDA-approved PC immunotherapy sipuleucel-T and the investigational vaccine Prostvac utilizes epithelial differentiation proteins (prostatic acid phosphatase and PSA, respectively) to prime dendritic and T cells, and results in only modest survival benefits [241, 242], tumor escape from this immunotherapy may involve EP and loss of epithelial targets or upregulation of mesenchymal or stemness targets.

As discussed in the clinical vignette above, one path to CRPC may be through neuroendocrine differentiation (NED), in which PC cells no longer secrete PSA or express AR. Instead, the cells often express and secrete chromogranin A [20], and this may be another example of EP. Clinically, NED most often occurs after ADT or AR signaling inhibition. Likewise, preclinically, depletion of androgen in cell culture promotes NED of PC cells [243], and NED in response to androgen deprivation in cell lines is dependent on Akt activity [62]. Given the known crosstalk between the AR and PI3K–Akt pathways [244], there is rationale for a combination approach clinically, and PI3K–Akt pathway inhibitors are currently under investigation both as single agents and in combination (reviewed in [245]). PC tumors with NED often have high levels of EZH2, which as discussed above, leads to suppression of DAP2IP and subsequent activation of important oncogenic pathways and EMT, further supporting the hypothesis that NED is a result of EP [28]. To further classify NED in PC, next-generation RNA sequencing was performed on primary tumors and metastatic biopsy samples from men with NED and compared with tumors from men with classic prostate adenocarcinoma. Aurora kinase A and N-myc are overexpressed and amplified in 40 % of NED versus 5 % of classic prostate adenocarcinoma and cooperate to induce NED in prostate cells [28]. This suggests that aurora kinase inhibitors may be used alone or in combination with cytotoxic chemotherapy to treat NED in PC, and trials targeting aurora kinase A are ongoing. Finally, whether NED is associated with EP or genetic evolution in PC is not clear. However, small cell differentiation of nonsmall cell lung carcinomas has been reported during EGFR inhibition, which is reversible phenotypically upon withdrawal of the epithelial targeting agent [22]. This suggests that a similar phenomenon may be occurring in PC during ADT or with potent AR inhibition.

4 Therapeutic strategies directed toward EP

As described above, there is substantial evidence that one mechanism of drug resistance is through phenotypic plasticity. In the era of personalized medicine, combination anticancer therapies have fallen somewhat out of favor; however, rational combination approaches may eradicate PC, similar to the way combination therapy revolutionized treatment for leukemia and infectious diseases, such as tuberculosis and HIV/AIDS. Combination strategies in preclinical models of malignancy have turned cytostatic activity into cytocidal activity and resulted in durable remissions. Therefore, combining therapies based on the knowledge of resistance pathways inherent to the cancer cell and the tumor microenvironment is an emerging and essential step in oncology [246]. We hypothesize that targeting the underlying regulators of EP, such as stemness pathways, epigenetic regulators, or oncogenic pathways, will be more effective than single agent therapies directed against more traditional epithelial differentiation pathways. These epithelial differentiation pathways, including AR and androgen synthesis, are not likely to be causally related to PC survival, given the inevitable resistant escape observed clinically after relatively short intervals of time. While a PC stem cell has not been clearly identified, it is possible that AR is not present in this stem-like cell [247], and thus, strategies to target key stemness, invasion, and dissemination pathways may be of greater benefit than AR targeting. However, given the central role of AR in PC and its potential role in promoting survival of PC cells, targeting AR in the context of additional therapies directed against EP regulators may remain critical. Indeed, multiple pathways may require targeting in order to address the bulk of the differentiated cancer and its stem-like progeny [200].

There are several available drugs and therapies in development that specifically target the epithelial or the mesenchymal phenotype or stemness pathways, and potential therapeutic approaches to addressing EP in the clinic are listed in Table 3. Agents directed toward epithelial targets, such as androgen synthesis and AR signaling inhibitors, may need to be partnered with therapy against mesenchymal targets for maximal benefit. For example, in cell lines with constitutively active AR variants, there is increased expression of mesenchymal markers including N-cadherin, again implicating EP as a mechanism of treatment resistance [248]. Furthermore,there is a monoclonal antibody against N-cadherin that, in mouse models, prevents invasion and metastasis and delays the time to castration-resistance [236]. Combining a pure mesenchymal target such as this with an epithelial target may be a rational approach, such as combinations with enzalutamide or abiraterone acetate. Epithelial-antigen immunotherapies such as Prostvac (against PSA) or sipuleucel-T (against PAP) may lead to mesenchymal or stemness-based immune escape, similar to what has been observed preclinically, and thus novel targeting of mesenchymal or stemness antigens may be more productive long term. In addition, targeting of stromal cells directly through prodrugs, monoclonal antibodies, or chemokine inhibitors may reduce EP and invasion indirectly [249].

Approaches that target embryologic pathways important in regulating EP may provide clinical benefits similar to those observed preclinically. For example, treatment with a cytotoxic agent such as docetaxel may reduce the bulk of disease, but disease relapse is inevitable. Activation of Hedgehog or

Table 3 Potential therapeutic strategies directed toward EP

Therapy	Mechanism of action	Efficacy in human PC	References
Epithelial phenotypic targets			
Androgen receptor antagonist			
Enzalutamide ARN-509 TOK-001	Blocks AR, targets epithelial cells	Enzalutamide prolongs survival; Multiple agents in phase II-III trials	[256–258]
Androgen synthesis inhibitors			
Abiraterone Orteronel TOK-001	Inhibits the CYP17 enzymes needed for testosterone synthesis, targets epithelial cells	Abiraterone prolongs survival; orteronel in phase II-III trials	[258–261]
Mesenchymal phenotypic targets			
N-Cadherin			
Anti-N-cadherin antibody ADH-1 (Exherin)	Block N-cadherin to slow tumor growth and inhibit EMT	Unknown	[235, 262]
Clusterin inhibition			
OGX-011 (custersin)	Antisense oligonucleotide against secretory clusterin, may inhibit EMT	OGX-011 in combination with docetaxel improved survival in a phase II of men with CRPC	[92, 93]
C-met			
Cabozantinib	Tyrosine kinase inhibitor against MET and VEGFR2	Bone scan and progression-free survival improvement	[219]
Sarasinoside A1	Induces MET, even in the absence of E-cadherin	Unknown	[263]
Stromal targets			
fibroblast specific protein (FSP)	Prodrug targets stroma and may prevent EMT	Unknown	[249]
FGFR family (mesenchymal isoforms)	Inhibits invasion, survival	Unknown	[264]
Aurora kinase A inhibitor (MLN8237)	Blocks neuroendocrine differentiation	MLN8237 in phase II trials	[28]
Combination approaches			
Immunologic therapies in combination			
Checkpoint/vaccine strategies	Target multiple antigens during escape from initial immunotherapy	Unknown	[240]
Epigenetic therapies in combination			
HDAC inhibitors	Induce EMT or MET	Unknown	[156]
Stemness pathway targets			
TGF-β pathway inhibitors	Kinase inhibition, neutralizing antibodies, or antisense oligonucleotides	Unknown	[252]
Hedgehog/Gli signaling inhibitors	Small molecule inhibition of Gli	GDC-0449 in phase 1-2 trials	[248]
NOTCH inhibitors	Gamma secretase inhibition	Unknown, ongoing	[251]
PI3K/PTEN pathway inhibitors	Reduced stemness, survival	BKM120, others in phase 1-2 trials	[245]

PSA prostate specific antigen, *ADT* androgen deprivation therapy, *CRPC* castration-resistant prostate cancer

NOTCH signaling in CRPC patients suggests that biomarkers of stemness may predict for benefit of agents that block stemness pathways. Hedgehog and NOTCH signaling inhibition is an active area of investigation in prostate and other cancers, and clinical trials with these agents alone and in combination are ongoing (reviewed in [250, 251]). Combination therapy with Hedgehog or NOTCH inhibition to address the stem-like cells with loss of epithelial differentiation may be more effective than treatment with either agent alone [200]. However, investigation of the selectivity of these agents against tumor cells rather than normal hematopoietic and organ-specific stem cell niches will be imperative given the potential for stem-cell toxicity. In a high-throughput drug screen to uncover agents specific to EMT-induced stemness properties, there were only a handful of agents, such as salinomycin, that were specifically toxic to cancer stem cells over normal cells, illustrating the formidable problem of selectivity. In this screen, paclitaxel actually induced a greater metastatic burden and promoted stemness properties [204]. These surprising findings require further

validation in PC model systems, where new classes of agents more specific to the underlying biology of EP rather than differentiated cells may bear greater fruit.

Another stemness target under investigation is TGF-β and the differing roles of TGF-β in early versus late stage cancer and in mediating hematogenous versus lymph node metastases, as described above, highlights the need for biomarkers to help guide patient selection for treatment with these agents. Clinical trials with anti-TGF-β therapies will likely show different results depending on the clinical context and again may be more effective when given in combination (reviewed in [252]). A clearly defined biomarker or set of biomarkers for EP in PC is needed to track these phenotypically diverse cells as they progress and contribute to treatment resistance. For example, detection of AR variants may be predictive of treatment response or resistance [253]. As reviewed elsewhere, predictive biomarkers in CRPC require extensive validation and prospective qualification both preclinically and in clinical trials, before they can be incorporated into clinical practice [254]. AR-independent PC may also be important in the development of EP, and identifying biomarkers of the different PC disease states and their relationship with EP is crucial.

Finally, because disease stability and differentiation rather than rapid cytoreduction and tumor shrinkage may occur with these therapies, especially when investigated as single agents, clinical trial endpoints that adequately test the activity of antiplasticity or stemness agents are necessary. In CRPC, these endpoints may include metastasis-free survival, overall survival, and radiographic or clinical progression-free survival. Combination approaches leading to novel cure model based clinical trial designs would also provide fair tests of substantial long term activity while limiting sample size [255], similar to what has been observed in the treatment of tuberculosis and HIV infections. Thus, combination approaches of EP targeted therapy with more traditional hormonal, immunomodulatory, or chemotherapies may extend survival, similar to what has been observed preclinically.

5 Conclusions

Substantial improvements in outcomes have been realized with novel hormonal therapies used for the treatment of metastatic CRPC, including abiraterone acetate and enzalutamide, and with immunotherapies and chemotherapies, such as sipuleucel-T, docetaxel, and cabazitaxel. Despite these incremental advances, treatment resistance emerges within 1–2 years in most cases, suggesting that novel approaches are needed. With the clinical use of more potent androgen pathway inhibitors, the emergence of neuroendocrine and other variant phenotypes is predicted to rise. EP is clearly associated with dissemination in multiple solid tumors, and emerging evidence supports EP as a mediator of both hematogenous dissemination (bone, visceral metastases) and therapeutic failure. To address this biology, novel agents that target stemness and embryonic pathways that influence cellular differentiation and invasion will be needed, likely in combination with current therapies that target the more differentiated epithelial bulk of the metastatic lesions. Rational combination therapies, based on the knowledge of feedback resistance pathways inherent to the cancer cell and tumor microenvironment, as well as on knowledge of immunologic escape due to loss of epithelial antigens, will likely be the most effective way to target EP in PC.

6 Key unanswered questions

1 How is AR regulation related to EP in PC and are these two pathways linked?
2 Can metastasis occur in human PC without loss of an epithelial phenotype or gain of a mesenchymal phenotype? Can other forms of migration/invasion, such as amoeboid invasion or collective sheet migration also explain dissemination and treatment failure?
3 Does EP explain treatment resistance to enzalutamide and abiraterone acetate or immunotherapy with sipuleucel-T based on studies of CTCs and metastatic biopsies over time in patients?
4 Can combination approaches targeting both epithelial and stem-like/mesenchymal compartments lead to eradication of established metastases or are these approaches more effective at preventing metastatic disease?
5 Does secondary neuroendocrine PC emerge due to genetic evolution and clonal selection over time or can this phenotype be reversed through systemic therapies, implying cellular plasticity?

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